

**Functional Characterization of Transgenic *Arabidopsis thaliana*
Plants Co-over expressing Aldehyde dehydrogenases and Genes for
Soluble Osmolytes**

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ABBREVIATIONS

ABA	Abscisic acid
ALDH	Aldehyde dehydrogenase
Amp	Ampicillin
APS	Ammonium persulfate
BA/BAP	Benzly Adenine/Benzyl Amino Purine
BSA	Bovine serum albumin
bp	Base pairs of nucleotides
Bq	Becquerel
CaMV	Cauliflower Mosaic Virus
CTAB	Cetyltrimethylammoniumbromide
2,4-D	2,4-Dichlor phenoxy acetic acid
D	Days
Da	Dalton
DAB	3,3'-diaminobenzidine
DATP	Desoxy-adenosin-triphosphate
DCTP	Desoxy-cytidin-triphosphate
DGTP	Desoxy-guanosin-triphosphate
DMF	Dimethylformamid
DMSO	Dimethylsulfoxide
DNA	Desoxyribo Nucleic Acid
dNTPs	Desoxy-nucleotide triphosphate
DTT	1,4 Dithiothreitol
dTTP	Desoxy-thymidin-triphosphate
EDTA	Ethylenediaminetetraacetate
g	a) grams (unit of weight) b) as units of centrifugal force (9.81 m/s^2)
GA	Gibberelins
GST	Gluthation-S-transferase
hr(s)	Hour(s)
HEPES	4-(2-Hydroxyethyl)-1-piperazinethansulfonic acid
IAA	Indole Acetic Acid
IBA	Indole Butyric Acid
IPTG	Isopropyl- β -D-thiogalactopyranoside

Kana	Kanamycin
M	Molar, mole per litre
MCS	Multiple cloning site
min	Minutes
MOPS	3-(N-morpholino) propanesulfonic acid
NAA	Nepthaline Acetic Acid
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PIPES	Piperazine-N,N,-bis (2-ethanesulfonic acid)
PVP	Polyvinylpyrrolidon
Rif	Rifampicin
RNA	Ribo Nucleic Acid
RNase	Ribonuclease
RT	Room temperature
sec	Seconds (units of time)
SDS	Sodium dodecyl sulfate
SSC	Saline sodium citrate buffer
T _m	Melting temperature of primer
TAE	Tris Acetate EDTA
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TEMED	N,N,N,N-tetramethylethylenediamine
Tris	Tris-(hydroxymethyl)-aminomethane
Triton X-100	Poly(ethyleneglycolether)n-octylphenol

Summary

Plant growth and productivity is largely hampered by a number of abiotic stresses. These stresses affect the physiological and metabolic pathways leading to the production of many harmful substances like aldehydes, reactive oxygen species and abscisic acid etc. Aldehyde dehydrogenases (ALDHs) are important in detoxification of potentially toxic aldehydes in many plants. They are intermediates in a variety of fundamental biochemical pathways, but they can also be generated in a variety of environmental stresses like salinity, dehydration and temperature extremes. Transgenic *Arabidopsis* plants containing two different aldehyde dehydrogenases (ALDH3I1 and ALDH7B4) were characterized under various stress conditions. RNA and proteins were extracted; RT-PCR and Western blots were done to confirm the expression of the genes. Western blots revealed that in almost all of the salt (200 mM NaCl) as well drought stressed plants the ALDH3I1 and ALDH7B4 specific proteins were expressed. The RT-PCR also showed higher expression levels of mRNAs, of the two aldehyde dehydrogenase genes in the stressed plants as compared to the wild type (untransformed) *Arabidopsis* plants. In addition to molecular studies some physiological studies i.e., measurement of rate of gas exchange/photosynthesis and measurement of lipid peroxidation, were also done. After one week of stress treatment the CO₂ assimilation rate was measured using GFS-3000 (Heinz WALZ GmbH). The transformed plants showed a higher CO₂ assimilation rate than wild-type plants under both drought and salt stress. Similarly, lipid peroxidation was higher in wild-type *Arabidopsis* plants as compared to transgenic single and double over expressor plants under stress conditions. Based on these results it was decided to transform these genes into tobacco to investigate whether the same performance of the transgenic plants was seen. Transgenic tobacco plants as well as non transgenic plants were put under various stress conditions. The constitutive or stress activated expression of the gene resulted in increased tolerance to salt, drought and oxidative stress in transgenic tobacco plants associated with improved plant growth. It is demonstrated that transgenic plants produce less reactive oxygen species (ROS) under stress conditions; therefore, less damage occurs to cell structures specially membranes. Similar results were observed for the chlorophyll content of stressed plants that ultimately resulted in more photosynthesis and gas exchange. Malondialdehyde (MDA) that results from lipid peroxidation was also found to be at lower levels in transgenic tobacco plants under stress conditions. The transgenic plants accumulated higher amounts of proline than wild-type plants under drought stress. The studies suggest that *Arabidopsis* ALDH overexpression generally induces higher stress tolerance in tobacco plants. In addition to experiments with

ALDH genes, cloning of the genes responsible for production of the soluble osmolyte ectoine (1, 4, 5, 6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) was done. Genes of the ectoine synthesis were derived from a halophilic bacterium *Marinococcus halophilus*. Vector was designed in a way that all the three genes responsible for ectoine production possess different promoters and terminators; eventually the three genes were cloned in the same binary vector. The genes responsible for the synthesis of the soluble osmolyte ectoine were transformed into *Arabidopsis*. The ectoine genes showed variable degree of expression in the transgenic plants. The plants over-expressing all the three genes also produced ectoine but the amount was very low and could not be quantified under control conditions.

1 Introduction

1.1 Aldehyde Dehydrogenases (ALDH) Genes

Plant productivity is dependent on various biotic and abiotic factors. Unpredictable weather conditions increase the exposure of plants to temperature extremes, salinity, drought and massive rains. If one stress is followed by another; for example, long drought seasons followed by heavy rains can cause extensive damages which often results in the outburst of pathogens, pests and diseases, ultimately decreasing or destroying agricultural productivity (Rosenzweig *et al.*, 2001). The rapidly increasing population is also inserting a proportional pressure on utilization of resources and this pressure is further increasing due to industrialization. About 75% of water consumed globally is by the agricultural sector (Molden, 2007). For crop production water is one of the most important inputs and because of competition from industry the water available for agriculture. Crops are voracious consumers; for example, for paddy rice 5000 L of water is needed to produce 1 kg of grain. The major agricultural regions have been under the threat of drought due to lack of rain and more usage of water by other sectors. Thus, agricultural productivity worldwide is subject to other increasing environmental constraints, particularly to drought and salinity due to their high magnitude of impact and wide distribution. Currently 397 Mha, almost one third of the cultivated area, is affected by salts (FAO, 2000). Traditional breeding programs trying to improve abiotic stress tolerance have had some success, but are limited by the multigenic nature of the traits, complexity of genomes, longer crop cycles, dependence of seasons and many more factors. Using molecular techniques for genetic enhancement of stress tolerance represents an additional and cost-effective method for ensuring sustainable and improved crop yields in a variable and changing climate. A lot of measures have been tried and adopted to make plants tolerate abiotic stresses. Among all these use of biotechnological tools can produce plants which perform better under drought as compared to traditional breeding.

In order to understand the molecular and physiological background of stress tolerance of plants extensive studies are being carried out in a variety of plant species (Sunkar *et al.*, 2003; Zhang *et al.*, 2005; Feng *et al.*, 2008; Schramm *et al.*, 2008). The ability to accumulate or excrete selective ions, the control of ion absorption and translocation to the shoots, selective fluid transport from the xylem to other parts of the plant, ion accumulation

up on osmotic adaptation, ion intracellular compartmentation, organic solute accumulation, macromolecules protection, membrane lipids homeostasis, and function of membrane systems in saline environments are important cellular strategies, which may confer plant salt tolerance (Franco and Melo, 2000).

1.1.1 Ion Homeostasis

Salt stress disrupts plant ion homeostasis, resulting in excess toxic Na^+ in the cytoplasm and a deficiency of essential ions such as K^+ . When salinity results from an excess of NaCl , which is by far the most common type of salt stress, the increased intracellular concentration of Na^+ and Cl^- ions is deleterious to cellular systems (Serrano *et al.*, 1999). In addition, the homeostasis of not only Na^+ and Cl^- , but also K^+ and Ca^{+2} ions is disturbed (Hasegawa *et al.*, 2000; Rodriguez-Navarro, 2000).

1.1.2 Reactive Oxygen Species

Plants experiencing various stress conditions synthesize reactive oxygen species (ROS) that directly damage cellular components, such as membranes and inhibit photosynthesis (Price and Hendry, 1991). Increasing evidence has indicated that much of the injury to plants due to various environmental stresses is associated with oxidative damage through direct or indirect formation of ROS. The ROS, including superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^\cdot) and singlet oxygen (O^2), are inevitable by-products of cell metabolism. These ROS react with lipids, proteins and nucleic acids, causing lipid peroxidation, protein denaturation and DNA mutation.

Under physiological conditions, the production and destruction of ROS is balanced in the cell metabolism. However, under stress conditions, the formation of these radicals might be in excess of the amount present under physiological conditions, thus causing oxidative stress (Yu and Rengel, 1999). It seems likely that ROS, which are synthesized by plants experiencing various stress conditions, lead to damage. It also seems likely that ROS are scavenged by compatible solutes, resulting in the protection of plants against stress conditions (Smirnoff, 1993).

1.1.3 Transcription Factors

The biology of plants is in many aspects common to that of other organisms: there are, however, a number of biological processes that are unique to plants, e.g. photosynthesis, the reproductive process, development and responses to environmental signals. In the past decade, hundreds of transcription factors have been identified in plants that are involved in

the regulation of many biological processes. Their protein structures suggest that plants have in some cases adopted pre-existing prototype functional motifs and modified them for specific regulatory processes. Indeed, most of the functional motifs present in eukaryotic transcription factors have their counterparts in the plant kingdom. In the other cases, plants seem to have evolved new classes of functional motifs that are not present in other organisms. The modified and new functional motifs are considered to have coevolved with regulatory processes that are unique to plants (Takatsuji, 1998). Transcriptional modulation has always been predicted to play a major role in the control of plant responses to salt stress. Transcription factors have been identified based on interaction with promoters of osmotic/salt stress-responsive genes. These factors participate in the activation of stress-inducible genes, and presumably lead to osmotic adaptation. Since the promoters that are controlled by these transcription factors are responsive to several environmental signals, it is not clear which transcription factors, if any, function only in salt stress responses, or if salt-specific transcriptional regulation alone is a requisite component of salt tolerance *in planta* (Liu *et al.*, 1998; Sheen, 1996; Shinozaki and Yamaguchi-Shinozaki, 1996; Winicov and Bastola, 1999; Zhu *et al.*, 1997).

1.1.4 Stress Signalling

The process by which plant cells sense the stress signals and transmit them to the cellular machinery to activate adaptive responses is referred to as signal transduction. A multitude of exogenous stimuli, like light, temperature, nutrient availability, needs to be perceived and processed simultaneously to achieve an integrated response ensuring optimal adaptation to the environment (McCarty and Chory, 2000). Despite decades of physiological and molecular effort, knowledge of how plants sense and transduce low temperature, drought and salinity signals are still very limited (Xiong and Zhu, 2001). Regulatory molecules are signal transduction pathway components that control the amount and timing of these effector molecules.

Although ABA has broad functions in plant growth and development, its main function is to regulate plant water balance and osmotic stress tolerance. It also has regulatory roles in cell cycle and other cellular activities. The role of ABA in drought and salt stress is at least twofold: water balance and cellular dehydration tolerance. Whereas the role in water balance is mainly through guard cell regulation, the latter role has to do with induction of genes that encode dehydration tolerance proteins in nearly all cells (Zhu, 2002). The role of ABA in abiotic stress signaling is not straightforward. Not all stress signaling pathways

employ ABA and the entangled relation of ABA and stress signaling is obscure. The studies up to now have suggested that ABA-dependent and ABA-independent osmotic and cold stress pathways might converge at several hitherto unexpected points. If they do, this increases opportunities for coordination between stress signals and ABA in the regulation of gene expression. Osmotic stress induced ABA accumulation is a result of both activation of synthesis and inhibition of degradation. It is evident that ABA biosynthesis is subjected to osmotic stress regulation at multiple steps. To date, genes responsible for ABA degradation have not been identified. Nothing is known about the signaling between osmotic stress perception and the induction of ABA biosynthesis genes. Presumably, it involves calcium signaling and protein phosphorylation cascades (Zhu, 2002). Promoters of ABA-dependent osmotic stress-responsive genes include regulatory elements that interact with basic leucine-zipper motif (bZIP), MYB, or MYC domains in DNA binding proteins (Abe *et al.*, 1997, Shen and Ho, 1995, Shinozaki and Yamaguchi-Shinozaki, 1997). Transcription factors are supposed to play an important role in osmotic/salt stress gene induction, independent of ABA e.g., dehydration response element (DRE) binding proteins. Two gene families have been characterized, DREB1 and DREB2. Both, DREB1 and DREB2 family members also have domains that bind ethylene-responsive elements (Stockinger *et al.*, 1997).

1.1.5 Aldehydes

Aldehydes are intermediates in a variety of fundamental biochemical pathways, but they can also be generated in a variety of environmental stresses like salinity, dehydration and temperature extremes. Aldehydes are common by-products of a number of metabolic pathways, including the metabolism of vitamins, amino acids, carbohydrates, and lipids (Schauenstein *et al.*, 1977). The resulting aldehydes are highly reactive molecules; because of its electrophilic nature, through the aldehyde's carbonyl group, they can react with cellular nucleophiles, including proteins and nucleic acids. The damaging effects of aldehydes have been well studied in humans; they include cytotoxicity, mutagenicity, and carcinogenicity (Schauenstein *et al.*, 1977). Therefore, the removal of aldehydes is essential for cellular survival. Aldehyde dehydrogenases (ALDHs) represent a protein super family of NAD(P)⁺ dependent enzymes that oxidize various aldehydes to their corresponding carboxylic acids. Therefore active ALDH enzymes are an important pathway for the detoxification of aldehydes. In mammals and yeasts, at least two isozymes of ALDH are known to be involved in ethanol metabolism, cytosolic ALDH1 and

mitochondrial ALDH2 (Hsu *et al.*, 1988, 1989). In 1996, the first gene encoding a plant mitochondrial ALDH, the restorer of fertility 2 gene (*rf2*), was identified in maize (Cui *et al.*, 1996). This gene was later found to be a nuclear restorer gene of Texas-type cytoplasmic male sterility (cms-T) (Schnable and Wise, 1998). Subsequently, in tobacco, two *ALDH* genes (*ALDH2a* and *ALDH2b*) were identified, and the *ALDH2a* transcript and ALDH2a protein were found to be present at high levels in floral organs such as stamens,

Table .1. The ALDH family in *Arabidopsis thaliana* (Kirch *et al.*, 2005)

	Gene annotation	Fromer name	Locus	Gene bank accession number	ORF (bp)	aa	MW (KDa)	Putative subcellular localization	Putative function
Family 2	ALDH2B4	ALDH2a	At3g4800	AF349447	1716	538	58.6	mitochondria	Mitochondrial ALDH
	ALDH2B7	ALDH2b	At1g23800	AF348416	1605	534	58.1	mitochondria	Mitochondrial ALDH
	ALDH2C4	ALDH1a	At3g24503	AF349448	1506	501	54.3	cytosol	Cytosolic ALDH phenylpropanoid pathway (ferulic acid, sinapic acid biosynthesis)
Family 3	ALDH3F1	ALDH5	At4g36250	AJ584644	1452	483	53.5	cytosol	Variable substrate ALDH
	ALDH3H1	ALDH4	At1g44170	AY072122	1455	484	53.2	cytosol	Variable substrate ALDH
	ALDH3I1	ALDH3	At4g34240	AJ306961	1653	550	60.2	chloroplast	Variable substrate ALDH stress regulated detoxification pathway
Family 5	ALDH5F1	SSADH1	At1g79440	AF117335	1587	528	56.6	mitochondria	Succinic semi-aldehyde dehydrogenase stress regulated detoxification pathway
Family 6	ALDH6B2	MMS-ALDH	At2g14170	NM126989	1824	607	65.9	mitochondria	Methylmalonyl semialdehyde dehydrogenase
Family 7	ALDH7B4	ALDH6	At1g54100	AJ584645	1527	508	54.2	nd	Turgor response
Family 10	ALDH10A8	PutBADH	At1g74920	AY093071	1506	501	54.4	nd	Betaine aldehyde dehydrogenase
	ALDH10A9	PutBADH	At3g48170	AF370333	1512	503	54.9	mitochondria	Betaine aldehyde dehydrogenase
Family 11	ALDH11A3	GAPDH	At2g24270	AY037205	1491	496	53.1	nd	Non-phosphorylating glyceraldehyde 3-phosphate dehydrogenase
Family 12	ALDH12A1	P5CDH	At5g62530	AY039787	1671	556	61.7	mitochondria	D-pyrroline-5-carboxylate dehydrogenase, protection from proline toxicity
Family 22	ALDH22A1	ALDH7	At3g66658	AJ584646	1791	596	66.0	secretory pathway	Novel ALDH

pistils and pollen (Camp and Kuhlemeier, 1997). In rice two different types of the ALDHs were reported; one was found to be a cytosolic ALDH isozyme (ALDH1a) that exists in rice roots (Li *et al.*, 2000) and the other is found to be in mitochondria of dark grown seedlings, mature leaf sheaths and panicles (Nakazono *et al.*, 2000). Interestingly expression of the rice *Aldh2a* gene, unlike the expression of the tobacco (*Nicotiana tabacum*) *Aldh2a* gene, was induced in rice seedlings by submergence.

One of the big advantages of *Arabidopsis* is its completely sequenced genome which provides an invaluable tool to study the organization and molecular relationships of ALDH genes in detail in a plant. Some of the ALDH genes are being characterized in the group of D. Bartels (Bartels and Souer, 2003). It was previously shown that one member of this ALDH gene family confers stress tolerance, when it is over-expressed in *A. thaliana* (Sunkar *et al.*, 2003). *Arabidopsis thaliana* contains 14 different ALDH genes (Table 1, Kirch *et al.*, 2004) consisting members from 9 ALDH families; 8 families of them are believed to be known and 1 family is considered as a novel family (ALDH22). Some studies have been carried out on ALDHs from *Arabidopsis*. The genes ALDH3I1 and ALDH3H1 encode two novel aldehyde dehydrogenases belonging to class-3 ALDHs (Kirch *et al.*, 2001). The overexpression of ALDH3I1 in transgenic *Arabidopsis* plants leads to improved tolerance under diverse environmental stresses (Sunkar *et al.*, 2003). ALDH5F1 (SSADH1) encodes a succinic semialdehyde dehydrogenase; localized in mitochondria and constitutes a member of the γ -aminobutyric acid (GABA) shunt in *Arabidopsis* (Busch and Fromm, 1999). T-DNA mutants of SSADH1 are dwarf plants with necrotic lesions and display an enhanced sensitivity to both UV-B light and heat stress which is correlated with increased H₂O₂ levels, suggesting that this gene restricts levels of reactive oxygen species (ROS) intermediates in plant defence against environmental stress (Bouche *et al.*, 2003). Recently it has been demonstrated that constitutive or stress-inducible expression of the chloroplastic *ALDH3II* and the cytoplasmic *ALDH7B4* confers tolerance to osmotic and oxidative stress. Stress tolerance in transgenic plants was accompanied by a reduction of H₂O₂ and malondialdehyde (MDA) derived from cellular lipid peroxidation (Kotchoni *et al.*, 2006).

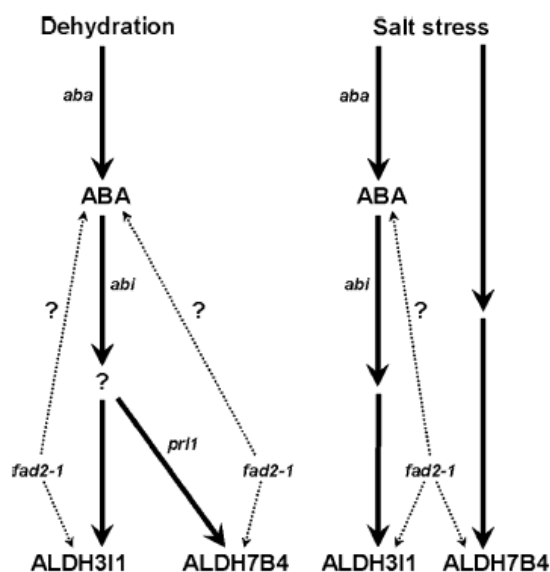


Fig. Proposed model of putative pathways involved in ALDH3I1 and ALDH7B4 expression in Arabidopsis. ABA regulates dehydration stress response of both genes, and mediates salt stress responsive expression of ALDH3I1. PRL1 is a positive factor of the ALDH7B4 dehydration response, while an altered membrane lipid composition (*fad2-1*) interferes with osmotic stress response of ALDH3I1 and ALDH7B4 in an ABA-regulated manner in an as yet unknown way. (Kirch *et al.*, 2005)

In addition to ALDH there are also reports of ALDH related genes (e.g. Betaine aldehydedehydrogenase BADH) cloned from several plants (Weretilnyk and Hanson, 1990 & 1992; Xiao *et al.*, 1995; Legaria *et al.*, 1998; Hibino *et al.*, 2001) and transferred into tobacco (Rathinasabapathi *et al.*, 1994), wheat (Guo *et al.*, 2000) and watercress (Li *et al.*, 2000). The expression of BADH in those plants resulted in salinity tolerance.

1.2 Soluble Osmolytes, Ectoine genes

One way many plants and other organisms cope with osmotic stress is to synthesize and accumulate compounds termed osmoprotectants (soluble osmolytes or compatible solutes). These are small, electrically neutral molecules that are non-toxic at molar concentrations and stabilize proteins and membranes against the denaturing effect of high concentrations of salts and other harmful solutes (Yancey, 2005). Chemically, osmoprotectants are of three types (1) betaines (fully N-methylated aminoacid derivatives) and related compounds such as dimethylsulfoniopropionate (DMSP) and choline-*O*-sulfate, (2) certain amino acids like proline and ectoine, and (3) polyols and non reducing sugars such as trehalose. Not all of these occur in crop plants naturally. However, an important feature of osmoprotectants is that their beneficial effects are generally species unspecific, so that a foreign osmoprotectant can be engineered into any plant and protect their new host (Rontein *et al.*, 2002). Transgenic plants harbouring genes for biosynthesis of mannitol (Tarczynski *et al.*, 1993), proline (Kishor *et al.*, 1995), ononitol (Sheveleva *et al.*, 1997), trehalose (Romero *et*

al., 1997), betaine (Hayashi *et al.*, 1997; Sakamoto *et al.*, 1998; Moghaieb *et al.*, 2000) and fructan (Pilon-Smits *et al.*, 1995) have shown marked improvement in water stress tolerance. In addition to their role in the adaptation of organisms to high-osmolality habitats, compatible solutes also have protein-stabilizing properties (Arakawa *et al.*, 1985) that support the correct folding of polypeptides under denaturing conditions both *in vitro* (Arora *et al.*, 2004) and *in vivo* (Barth *et al.*, 2000; Chattopadhyay *et al.*, 2004; Ignatova and Gierasch 2006). These properties most likely result from unfavorable interactions of compatible solutes with the protein backbone (Bolen and Baskakov, 2001; Street *et al.*, 2006) and the concomitant preferential exclusion of these compounds from the immediate hydration shell of proteins (Arakawa *et al.*, 1985). The stabilizing properties of compatible solutes probably also contribute to their function as microbial stress protectants against heat stress (Bursy *et al.*, 2008; Canovas *et al.*, 2001; Purvis *et al.*, 2005), salt stress (Canovas *et al.*, 1997; Bursy *et al.*, 2008) and chill stress (Angelidis and Smith 2003; Brigulla *et al.*, 2003; Kuhlmann *et al.*, 2008). Since compatible solutes function as protein stabilizers under various types of stress conditions (Knapp *et al.*, 1999) they are also sometimes referred to in the literature as chemical chaperones (Diamant *et al.*, 2001; Chattopadhyay *et al.*, 2004). Compatible solutes also interact in various ways with nucleic acids (Kurz, 2008; Schnoor *et al.*, 2004) and can even influence protein-DNA interactions (Pul *et al.*, 2007). The compatible solutes that accumulated differ among plant species and can include betaines and related compounds; polyols and sugars, such as mannitol, sorbitol, and trehalose; and amino acids, such as glutamic acid and proline (Rhodes and Hanson, 1993; McNeil *et al.*, 1999; Serrano, 1999). In plant cells, osmoprotectants are typically confined mainly in cytosol, chloroplasts and other cytoplasmic compartments that together occupy 20% or less of the volume of mature cells (Rhodes and Samaras, 1994).

Compatible solutes are typically hydrophilic, which suggests they could replace water at the surface of proteins, protein complexes, or membranes, thus acting as osmoprotectants and non-enzymatically as low-molecular-weight chaperons. The physicochemical basis of this protective effect involves the exclusion of osmoprotectant molecules from the hydration sphere of proteins (Timasheff, 1992). This creates a situation in which native protein structures are thermodynamically favored because they present the least possible surface area to the water. In contrast, salts enter the hydration sphere and interact directly with protein surfaces, favoring unfolding. In dry or saline environments osmoprotectants can therefore serve both to raise cellular osmotic pressure and to protect cell constituents

(Rontein *et al.*, 2002). Osmoprotectants are thought to mediate osmotic adjustment, protecting sub-cellular structures and oxidative damage by their free radical scavenging capacity (Hong *et al.*, 1992; Smirnov, 1993; Hare *et al.*, 1998). Thus, genes regulating the accumulation of these organic compounds can be considered as salt tolerance determinants. Metabolic engineering is generally defined as the redirection of one or more metabolic pathways (enzymatic reactions) to produce new compounds in an organism, improve the production of existing compounds, or mediate the degradation of compounds (DellaPenna, 2001). Metabolic engineering of osmoprotectant pathways works for model plants subjected to more or less artificial laboratory tests of stress resistance. But there is a long path ahead to raise accumulation levels, to overcome intracellular transport constraints, to restrict accumulation to when and where it is needed, to reduce side-effects, and to prove the value of engineered osmoprotectants in major crops under field stress conditions (Rontein *et al.*, 2002).

Genetic transformation has allowed the introduction of new pathways for the biosynthesis of various compatible solutes into plants, resulting in the production of transgenic plants with improved tolerance to stress (McNeil *et al.*, 1999). Plants engineered to synthesize and moderately accumulate a number of osmolytes showed marginally improved performance under abiotic stress conditions. The effects seen with modest increases in mannitol, fructans, trehalose, ononitol, glycine betaine, or ectoine and with strong increases in proline amount indicate that the purely osmotic contribution of these metabolites to stress tolerance may not describe their function completely, i.e. that the pathway leading to a particular osmolyte may be more important than accumulation *per se* (Bohnert and Shen, 1998; Hare and Cress, 1997; Jain and Selvaraj, 1997; Nelson *et al.*, 1998). Osmolytes are also believed to act in scavenging ROS since the levels of osmolytes in the transgenic plants are too low to be significant in osmotic adjustment (Shen *et al.*, 1997).

The production of transgenic plants that can accumulate various compatible solutes, in particular plants of model species such as *Arabidopsis* and tobacco, has allowed this stress defense mechanism to be extended to crop plants, such as rice, potato and sugar beet with varying degrees of success (Chen and Murata, 2002).

Fructan producing transgenic tobacco plants performed significantly better than controls under drought conditions, having a 55% more rapid growth rate, 33% greater fresh weight and 59% greater dry weight than wild type plants (Pilon-Smits *et al.*, 1995). Transgenic

beet plants also accumulated fructan to about 0.5% of their dry weight in both roots and shoots. Moreover, these transgenic beets grew significantly better under drought conditions than did wild-type plants (Pilon-Smits *et al.*, 1999). Tobacco and *Arabidopsis* plants do not usually contain mannitol. However, expression of *mtlD* gene for mannitol-1-phosphate dehydrogenase from *E.coli* in these two species resulted in the biosynthesis of mannitol. The mannitol-producing tobacco plants exhibited increased tolerance of high salinity (Tarczynski *et al.*, 1993). In the seeds of mannitol accumulating *Arabidopsis* plants, the concentration of mannitol reached 10 $\mu\text{mol g}^{-1}$ dry weight. Mannitol expressing seeds were able to germinate in medium supplemented with up to 400 mM NaCl, whereas control seeds ceased to germinate at 100 mM NaCl (Thomas *et al.*, 1995).

The increased level of proline significantly enhanced the ability of transgenic seedlings to grow in medium that contained up to 200 mM NaCl. The increased levels of proline also reduce the levels of free radicals in response to osmotic stress, as determined by monitoring the production of malondialdehyde (MDA). Suggesting that in addition to acting as an osmolyte, proline might play a role in reducing the oxidative stress that is brought on by osmotic stress (Hong *et al.*, 2000).

Holmström *et al.* (1996) transformed tobacco with the gene for the trehalose-6-phosphate synthase (TPS1) subunit of yeast trehalose synthase, which was driven by the promoter of the *rbcS* gene from *Arabidopsis*. The accumulation of trehalose seemed to improve drought tolerance but they exhibited a 30-50% reduction in growth rate. The transgenic tobacco plants expressing the same gene exhibited significantly enhanced tolerance to drought. However, as in the case of the transgenic tobacco plants that expressed TPS1, the transgenics exhibited various morphological changes which ranged from severely retarded growth to yellowish, lancet-shaped leaves and the aberrant development of roots (Yeo *et al.*, 2000).

1,4,5,6-Tetrahydro-2-methyl-4-pyrimidinecarboxylic acid (ectoine; a cyclic amino acid) functions as a compatible osmolyte in halophilic bacteria. Initially it was discovered as a minor component in the phototrophic sulfur bacterium *Ectothiorhodospira halochloris* (Galinski *et al.*, 1985). Since then ectoines (ectoine and its hydroxyl derivative, hydroxyectoine) are well known as the representative of ubiquitous compatible solutes serving mainly as osmoprotectants for halophilic and halotolerant bacteria under high osmolarity. Ectoine is synthesized by three successive enzymatic reactions from aspartic b-semialdehyde. The genes encoding the enzymes involved in the biosynthesis, *ectA*, *ectB*,

and ectC, encoding L-2,4-diaminobutyric acid acetyltransferase, L-2,4-diaminobutyric acid transaminase, and L-ectoine synthase, respectively, have been previously cloned in tobacco (Nakayama *et al.*, 2000).

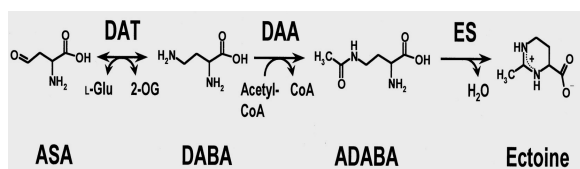


Fig.1. Ectoine synthesis pathway (Nakayama *et al.*, 2000). ASA=L-aspartate β-semialdehyde
DABA=L-2,4-diaminobutyric acid
ADABA=N⁷-acetyl-L-2,4-diaminobutyric acid

Ectoine like all soluble osmolytes, when present in the stressed cells tends to maintain the water level in the cells and retains turgidity and eventually cells can grow and perform their functions normally under osmotic unfavourable conditions (Record *et al.*, 1998). Recently ectoines have been paid attention as one of the most useful compounds to show a variety of characteristics such as stimulation on the respiration as well as growth of *Escherichia coli* (Nagata *et al.*, 2002) or heat resistance of enzyme activities (Zhang *et al.*, 2006), regular PCR enhancers by decreasing the melting temperature of dsDNA (Schnoor *et al.*, 2004), skin protection from UVA induced cell damage (Buenger and Driller, 2004), and inhibition of aggregation and neurotoxicity of Alzheimer's b-amyloid (Kanapathipillai *et al.*, 2005). Ectoine, when provided exogenously can reverse growth inhibition caused by osmotic stress in *Escherichia coli* (Jebbar *et al.*, 1992), *Corynebacterium glutamicum* (Farwick *et al.*, 1995), and the soil bacterium *Rhizobium meliloti* (Talibart *et al.*, 1994).

1.3 Objectives

The major objective of the study was to investigate whether the over-expression of more than one ALDH genes in plants increases the stress tolerance compared to the plants in which only one ALDH gene is over-expressed. For this purpose, Arabidopsis plants over-expressing two different ALDH genes were subjected to various abiotic stresses and were monitored by molecular and physiological signalling. In addition, it was tested whether over-expression of ALDH genes in tobacco could also improve stress tolerance. Moreover, ectoine genes - responsible for the production of the soluble osmolyte ectoine- from halophilic bacteria were cloned in a combinatorial fashion and ultimately transformed into Arabidopsis. Based on the molecular and physiological signalling the role of aldehyde dehydrogenases and ectoine genes will be discussed within the framework of genomics of abiotic stress responses.

2 Materials and Methods

2.1 Plant Material

Seventeen accessions of *Arabidopsis thaliana*; wild-type (Col 0), 2 lines with either ALHD3I1 (SA3P3) or ALDH7B4 (SA7P3) and 14 lines co-expressing the ALHD3I1 and ALDH7B4 were kindly provided by S. Kotchoni (IMBIO, Prof. Bartels, Uni-Bonn) and were used for characterization under control as well as stress conditions. The best performing lines were used for further stress treatments and characterization. In addition of ALDH *Arabidopsis* lines, transgenic plants containing soluble osmolyte ectoine were also created and used to characterize under various stress conditions.

2.1.1 *Arabidopsis thaliana*

Accessions of *A. thaliana* WT (Col 0), SA3P3, SA7P3, SA37P1, SA37P2, SA37P3, SA37P4, SA37P5, SA37P6, SA37P7, SA37P8, SA37P9, SA37P10, SA37P11, SA37P12, SA37P13 and SA37P44 were used.

Here ‘S’ means the plants were transformed with Cauliflower Mosaic Virus (CaMV) 35S promoter. ‘A’ stands for ALDH gene 3 or 7. Number immediately after A shows the ALDH 3I1 or/and ALDH7B4 gene. ‘P(n)’ stands for progeny lines where n=number assigned to various independent transgenic lines.

Floral dip method was also employed to get transformed plants of *A. thaliana* (Col 0) for ectoine genes.

2.1.2 *Nicotiana tabacum*

Tobacco (*Nicotiana tabacum*) SR1 plants transformed with the ALDH 3I1 and ALDH7B4 using *Agrobacterium* strain GV3101 mediated transformation of leaf discs. Initially thirteen independent transformants of ALDH3I1 and eleven independent transformants of ALDH7B4 were produced. WT (SR1), SA3P1, SA3P2, SA3P3, SA3P4, SA3P6, SA3P7, SA3P8, SA3P9, SA3P10, SA3P11, SA3P12, SA3P13 and SA3P14 were the lines with ALDH3I1 gene. For ALDH7B4 lines SA7P1, SA7P2, SA7P3, SA7P4, SA7P5, SA7P8, SA7P9, SA7P10, SA7P11, SA7P12 and SA7P13 were obtained. All the lines were tested via in vitro screening on MS medium (Murashige and Skoog, 1962) with various concentrations of NaCl (0, 200 mM and 400 mM). Few lines were selected for further stress treatments and characterization.

2.1.3 *Lycopersicon esculantum*

Tomato (*Lycopersicon esculantum*) cv. Money maker was used to transform as a part of ectoine genes cloning strategy.

2.2 Baterial Species and Strains

2.2.1 *E. coli* DH10B (Lorrow and Jessee, 1990)

Genotype: F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ 80d *lacZ*ΔM15 Δ *lacX74* *endA1* *recA1* *deoR* Δ (*ara, leu*)7697 *araD139 galU galK nupG rpsL λ*⁻

2.2.2 *E. coli* XL1-Blue (Stratagene, Amsterdam, NL)

Genotype: *endA1 gyrA96 hsdR17 lac recA1 relA1 supE44 thi-1*
[F⁺ *proAB lacI*^qZ ΔM15 Tn10 (Tet^r)]

2.2.3 *Agrobacterium tumefaciens* LBA 4404 (pAL 4404) (Ooms et al., 1982; Hoekema et al., 1983)

Genotype: Sm^r, (Rif^r) vir-region

2.2.4 *Agrobacterium tumefaciens* GV3101 (Holsters et al., 1980)

Genotype: C58-C1 (Rif^r) vir-region

2.3 Plasmid Vectors

pSAT4.35SP.MCS.35ST (DQ005466), pSAT6.rbcP.MCS.rbcT (DQ005473), pSAT7.actP.MCS.agsT (DQ005453) (Chung et al., 2005) were used as primary vectors for the three ectoine genes and binary vector pPZP 200 containing pRCS2-ocs-nptII (DQ005456) (Chung et al., 2005) was used to transform the plants with ectoine genes.

2.4 Primers (Sequences 5' → 3')

Name	Sequence from 5' to 3'	Purpose	Res. site
Ect A NcoI 5'	AGGTGGTAAGACCATGGAAACGA	point mut.	Nco I
Ect A SalI 3'	ACTTTGCATGTCATGTCGACTACT	point mut.	Sal I
Ect B NcoI 5'	AGGATAATTACTTCCATGGTGCAG	point mut.	Nco I

Ect B BamHI 3'	TCGGCTTCTTTGGATCCAATTAGT	point mut.	Bam HI
Ect C NcoI 5'	ATGGAGGAATAGACCATGGAAGT	point mut.	Nco I
Ect C HindIII 3'	TCACTCTAAAAAGCTTCAGGGGA	pointmut.	Hind III
Ect A SbfI 3'	ATGCTTCCTGCAGGTATGTTGTG	point mut.	Sbf I
Ect A SbfI 5'	GGATGTCCTGCAGGGCGATTAAG	point mut.	Sbf I
Ect B PacI 5'	CGATTAAGTTAATTAACGCCAG	point mut.	Pac I
Ect B PacI 3'	ATGACCTTAATTAATGGCAAACA	point mut.	Pac I
Ect C AflII 3'	GAAACACTTAAGACCATGATTAC	point mut.	Afl II
Ect C AflII 5'	AGTGCCCTTAAGGCCGTCAGTGTC	point mut.	Afl II
DQ 5'	ACCTCTGACACATGCAGCTC	Sequencing	No
DQ 3'	GCCTTTGAGTGAGCTGATAC	Sequencing	No
pPZP 5'	TCGCTCTTAGCCGTACAATA	Sequencing	No
pPZP 3'	AACTTAGGACTTGTGCGACAT	Sequencing	No
Ect A pcr 5'	ACAAACCAACCGTGGAAGAC	Sequencing	No
Ect A pcr 3'	TTCTTCGTGCTCGTCATCTG	Sequencing	No
Ect B pcr 5'	ATCTAGAAACCATGGTGCAGAATG	Sequencing	No
Ect B pcr 3'	CTAGGTGGATCCAATTAGTCATG	Sequencing	No
Ect C pcr 5'	AGATTTGCTCGGCACTGAAC	Sequencing	No
Ect C pcr 3'	ATGCACTTCACGACCGCTAA	Sequencing	No

2.5 Chemicals, Radioactive and Other Supporting Materials

The chemicals used in this study were purchased from Biomol (Hamburg, Germany), Difco BRL (Heidelberg, Germany), Merck VWR International (Darmstadt, Germany), PEQLAB (Erlangen, Germany), Perbio Science (Bonn, Germany), Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany), Sigma Aldrich (München, Germany) Qiagen (Hilden, Germany), Machery Nagel (Düren, Germany). The chemicals used were of analysis grade.

Radio-labelled [$\alpha^{32}\text{P}$] dCTP with starting activity of 370 MBq/ml was bought from Hardtmann (Germany).

Other materials like elektroporation cuvettes from Molecular BioProducts Inc. (San Diego, CA, USA) and filter papers from Whatman (GE Healthcare, UK).

2.6 Enzymes and Size Markers

Restriction endonucleases and their activity specific Buffers used were from Fermentas (St. Leon-Rot, Germany), New England BioLabs (UK), Amersham Pharmacia Biotech/Bioscience (Freiburg), Roche/Boehringer (Mannheim, Germany), Sigma-Aldrich (Germany) and Invitrogen/GibcoBRL (Karlsruhe, Germany).

For colony PCRs self prepared *Taq*- Polymerase (Pluthero, 1993) by Birgit Walldorf and Dr Nicolas Sauerbrunn was used. Amplification of ectoine genes and their subsequent subcloning into the primary and binary vectors was done using high fidelity *Taq* polymerase from Takara Bio Inc. (Shiga, Japan) and *Pfu* polymerase from Stratagene (La Jolla, CA, USA). 1 kb-Ladder from Fermentas was used as DNA marker while for proteins, the size marker was purchased from Sigma-Aldrich.

2.7 Membranes and Films

For RNA-Transfer either Hybond N or Hybond N+ membrane from Amersham Pharmacia Biotech was used. For southern blots (DNA transfer) and Western blots (protein transfers) Protran BA 85 (0.45 µm) from Schleicher & Schüll/Whatman (GE Healthcare, UK) was used. For both type of nucleic acids transfers, the required bands were first radio-labelled with radio active probe and then the signals were detected on the Phospho-imager screen (Amersham Biosciences).

2.8 Kits

DNAs were purified using Qiaex II Gel Extraction Kit, Midi- und Maxi-Plasmid purification kit from Qiagen (Hilden, Germany). Cloned ectoine genes were purified with Machenery Nagel Nucleo Spin Extract II purification kit. For subcloning ectoine genes and cassettes Clone Jet PCR cloning kit (Fermentas, Germany) was used. For labelling ³²P probes, Hexa LabelTM DNA Labeling Kit (Fermentas, Germany) was used.

2.9 Equipment and Instruments

The following equipment and instruments were used according to the manufacturers' instructions.

Elektroporation apparatus: Gene Pulser II with Pulse Controller II and Capacitance Extender II von Bio-Rad (Hercules, USA)

Particle Gun: PDS 1000/He System (Bio-Rad)

PCR-Maschine: Trio-ThermoblockTM 48 with heated lids (Biometra, Göttingen, Germany)

Photometer: Ultrospec 2000 (Amersham Pharmacia Biotech)

Photosynthesis: For gas exchange and chlorophyll fluorescence GFS-3000 (WALZ, Germany)

Typhoon Scanner (Amersham Pharmacia Biotech)

Conductivity meter: For electrolyte leakage conductivity meter set Qcond 2400 (VWR, Germany)

2.10 Softwares

For Sequence comparisons, translations, point mutagenesis and vector design the Programm Vector NTITM Suite 10.0 was used.

National Centre for Biotechnological Information web (www.ncbi.nlm.nih.gov/) was also used to compare the plasmid sequence.

2.11 Media, buffers and solutions

All media, buffers and solutions used were sterilized either by filter sterilization or by autoclaving for 20 min at 120 °C at 1.5 bars. Seedlings were grown on MS salts (Duchefa) and adding agar as described by Murashige and Skoog (1962).

2.11.1 Media

MS-medium (per litre): 4.6 g MS-salts, 20 g sucrose, 1 ml vitamins, adjust pH to 5.8 with 1M NaOH, and 8 g selected agar (optional).

LB-medium (per litre): 10 g peptone, 10 g NaCl, 5 g yeast extract, adjust pH to 7.5, and 15 g selected agar (optional) for agar plate cultures.

YEB (per litre):	5 g saccharose, 5 g of meat extract, 5 g peptone, 1 g yeast extract, 2 mM MgSO ₄ (0.493 g MgSO ₄), adjust pH at 7.0, and 15 g Select agar (optional) for agar plate cultures only.
SOC:	2% (w/v) trypton, 0.5% (w/v) selected yeast extract, 10 mM NaCl, 10 mM MgSO ₄ , 10 mM MgCl ₂

2.11.2 Buffers and solutions

10 x TAE buffer:	0.4 M Tris-acetate, 20 mM EDTA, pH 8.0.
RNase A + T1:	1mg/ml RNase A, 10000 U/ml RNase T1, 10 min heating and cooling at room temperature, and stored at -20°C for further use.
10 x blue gel loading buffer:	25 mg Bromophenol blue, 25 mg Xylencyanol, 1 ml 10 x TAE (as above), 3 ml glycerol, and 6 ml sterile distilled water (sd H ₂ O).
20 x SSC:	3 M NaCl, 0.3 M Sodium citrate, stored at room temperature.
1 x TE buffer:	10 mM Tris-HCl, 1mM EDTA, pH 8.0 and stored at room temperature.
Washing buffer:	0.1 % (w/v) SDS, 2 x SSC. Stored at room temperature.
MS-Vitamin solution:	2 mg/l glycine, 0.5 mg/l Niacin (Nicotine acid), 0.5 mg/l pyridoxin-HCl, 0.1 mg/l thiamine-HCl. 1:1000 dilution of the solution was used.

2.12 Culture of *Arabidopsis thaliana*

Seeds were sterilized in 70% (v/v) ethanol for 2 minutes and then in 0.6% NaOCl + 1-2 drops Triton X-100 per 100 ml for 10 to 15 minutes. During sterilization seeds were shaken continuously on a platform shaker. It was followed by 4 to 5 times rinsing with sterile distilled water and cultured on MS (Murashige and Skoog, 1962) agar plates with selection marker Kanamycin (50µg/ml). The plates were put in the growth room at 23°C and 18 hours light conditions. The plants were allowed to grow for 10 to 15 days on selection media (MS + Kanamycin) and then used for further stress analyses.

2.13 Tobacco Culture

Seeds of tobacco var. SR 1 were surface sterilized in 70% (v/v) ethanol for three minutes and then in 1% sodium hypochlorite in the presence of a few drops of Triton X 100 for a period of 10 to 15 minutes. During sterilization seeds were continuously shaken. It was followed by 3 to 4 times washing with sterilized double distilled water to remove traces of sterilizing agents. Seeds have been cultured on the MS medium and then put in a growth room at 25°C with 16 hours light conditions.

2.14 Tomato Culture

Seeds of tomato (*Lycopersicon esculantum*) var. Money Maker were surface sterilized with 70% (v/v) ethanol for 1 minute with continuous shaking. Then ethanol was replaced with 1.5 % sodium hypochlorite solution + 1-2 drops/ml of Triton X-100. The seeds were shaken similarly. Afterwards, the seeds were rinsed 4 to 5 times with sterile distilled water and cultured on 100ml ½ strength MS medium in magenta jars. The jars were wrapped in black paper and kept in growth room at 25°C.

2.15 Culture of *Eschericia coli*

E. coli cultures were raised at 37°C on LB agar plates with antibiotics (Ampicilin 50µg/ml or Spectinomycin 100µg/ml). In case of liquid cultures medium was in appropriate containers, size depending on culture volume, and shaken overnight at 225 to 250 rpm.

2.16 Culture of *Agrobacterium tumifaciens*

The cultures of *A. tumifaciens* were raised at 28°C in dark using YEB agar plates containing appropriate antibiotics (Spectinomycin 100µg/ml). The liquid cultures were shaken on a platform shaker at a speed of 225 to 250 rpm for 24 to 48 hours.

2.17 Plant genomic DNA extraction (Urea method)

Two to three leaves of *A. thaliana* (50-100 mg) were frozen in liquid nitrogen. Plant material was grinded to a fine powder in 2 ml eppendorf tubes using 3 big (2x5mm) and 2 small (2x3mm) metal beads. The vortex was pre cooled to support grinding and 2.0 ml

eppendorf tubes were used. Freshly prepared mixture of 375 μ l 2x lysis buffer and 375 μ l 2M urea solution were added to the plant material and vortexed for few seconds to homogenize it with plant material. Then 37.5 μ l phenol was added and vortexed briefly. The mixture was transferred into new 2.0 ml centrifuge tubes and kept on ice till other samples were undergone same step. Then 750 μ l of Phenol: Chloroform : Isomyl alcohol (25: 24: 1) mixture was added and centrifuged for 10 minutes at 13000 rpm at room temperature [25°C or room temperature (RT)]. Supernatants were taken into new tubes and 0.7 volume (500 μ l) of Isopropanol (RT) was mixed by gently inverting the tubes. Centrifugation was done for 30 minutes at 4°C and 13000 rpm. The supernatant was carefully discarded and pellets were washed with 1 ml 70% ethanol (2-3 minutes centrifugation). The ethanol was carefully discarded and pellets air dried for few minutes. The pellets were dissolved in 300 μ l Tris + RNase and kept at 37°C for 1-2 hours. At this stage the pellets was stored at 4°C overnight or addition of equal volume 300 μ l Phenol: Chloroform : Isomyl alcohol (25: 24: 1) followed by mixing gently and one more centrifugation for 5 minutes RT and 13000 rpm was done to eliminate the RNA. The supernatant was taken into a new 1.5 ml centrifuge tube and the DNA was precipitated (at least 1 hour -70°C) with 0.1 volume (30 μ l) 3M Sodium Acetate pH 5.2 and 2.5 volume (750 μ l) absolute ethanol. Final centrifugation was performed at 4°C for 20 to 30 minutes with 13000 rpm. Pellets obtained were washed with 70% ethanol, dried and finally dissolved in 40 μ l milli Q water

2x Lysis Buffer:

0.6 M NaCl
0.1 M Tris pH 8.0
40 mM EDTA
4% Sarcosyl
1% SDS

Tris + RNAase (1 μ l/ml of Tris)

RNAse Stock 100 mg/ml
Dilution (in 10 mM Tris pH 8.0) 1:1000

RNAase 100 μ l

Dissolve RNAse in sterile mQ H₂O
Boiled for 5 minutes and let cool to RT
Freeze at -20°C

Take care that RNase must not go inside micropipette

2.18 Plant genomic DNA extraction from *A. thaliana* (Doyle and Doyle, 1989)

500 mg plant material was grinded in liquid nitrogen. This ground plant material was homogenized with 7.5 ml of pre-warmed (60°C) DNA extraction buffer and kept at 60°C for 30 minutes.

DNA extraction buffer: 100 mM Tris HCl, pH 8.0
 20 mM EDTA
 1.4 M NaCl
 3.5 % (w/v) CTAB

This buffer can be stored at RT and before starting DNA extraction 0.2% β -Mercaptoethanol was added.

After keeping the mixture for 30 minutes at 60°C an equal volume (7.5 ml) of Chloroform: Isoamylalcohol (24: 1) was added and homogenized. The mixture was centrifuged at RT for 10 minutes 1600 g (5300 rpm) in Biofuge 15 R Heracus SEPATECH. The supernatant was transferred into new tubes and mixed with 2/3 volume (5ml) of pre-chilled Isopropanol and incubated at RT for 2 hours and centrifuged under the same conditions. Supernatants were carefully discarded, pellet air dried and resuspended in 1 ml 10 mM Tris HCl, pH 8.0. To this mixture 10 μ l RNase A+T i.e., 1 μ g/ μ l (boiled for 5 minutes before every time before use) was added and the mixture was incubated at 37°C for 30 minutes. Then 2 ml of 10 mM Tris-HCl pH 8.0, 1 ml of 7.5 M Ammonium Acetate pH 7.7 and 10 ml absolute ethanol were added and the solution was mixed gently by inverting the 15 ml falcon tube or glass tube and incubated at -70°C for at least 1 hour or overnight. The solution was centrifuged at 4°C 13000 rpm for 10 minutes (or 60 minutes at 4000 rpm when plastic falcon tubes were used). Pellet was washed twice with 70% (v/v) ethanol and air dried. After drying, the pellets were re-suspended in 50-100 μ l 10 mM Tris HCl pH 8.0 and stored at -20°C.

2.19 (Southern Blot) DNA transfer to Nitrocellulose Membranes (Southern, 1975)

For performing a Southern blot at least 20µg genomic DNA was digested overnight at 37°C with Eco RI for both Arabidopsis and tobacco lines. Then digested DNA samples were loaded on a 0.7% (w/v) agarose gel and run. The gel was briefly rinsed with distilled water and incubated in 250 mM HCl solution for 10-15 minutes without shaking, briefly rinsed with distilled water and put in denaturing buffer for 30 minutes with gentle agitation. The gel was again rinsed with distilled water and incubated under same conditions in neutralizing buffer. The gel was washed with distilled water and rinsed with 20 x SSC and blotted onto Protran BA 85-Membrane (Schleicher & Schüll). The blotting was done according to the standard capillary transfer protocol. DNA was bound to the membrane by exposing the membrane to UV for 2-3 minutes next morning or baked at 80°C for 1-2 hours.

Denaturing buffer: 1.5 M NaCl, 0.5 M NaOH without adjusting the pH
stored at room temperature.

Neutralizing buffer: 1 M Tris, 1.5 M NaCl, pH 8.0 (adjusted with
concentrated HCl) stored at room temperature.

2.19.1 Preparation of a $\alpha^{32}\text{P}$ -dCTP Radio Labelled Probe (Feinberg and Vogelstein, 1983)

The radioactive labelling was performed using Hexa Label™ DNA Labelling Kit (Fermentas) according to the instructions of the manufacturer. The method provides a good alternative to nick translation for producing uniformly radioactive DNA of high specific activity. The method relies on priming of the polymerase reaction on the template DNA with random hexanucleotide primers. The complementary strand is synthesized from the 3'-end of the primer with the help of the large fragment of DNA Polymerase I, Exonuclease minus (Klenow Fragment, exo-) in the presence of labelled deoxyribonucleoside triphosphates (contain dCTP). After the labelling reaction one of the nucleotide (dCTP) is radio labeled and un-incorporated dNTPs were removed by chromatography on Sephadex G-50. For this purpose 1ml syringe was blocked with a

GF/C-Filter and filled with 1 ml Sephadex G-50 resin in 1 x TE . The probe mix was passed through the column and 10 fractions of 100 μ l each of 1 x TE eluted. After measuring the counts of the fractions with the first peak was taken and denatured at 95°C for 5 minutes.

2.19.2 Hybridizing and Autoradiography

The membrane with nucleic acids was incubated in hybridizing buffer for at least 2 hours at 65°C in a water bath with shaker for pre-hybridizing. The buffer was renewed few minutes prior to the addition of denatured $\alpha^{32}\text{P}$ -dCTP radio labelled probe. The hybridization was performed overnight. And the next day the hybridizing buffer was removed and the membrane was washed to remove unspecific and over-bound probe. The washing was repeated 2 to 3 times (20 to 30 mins each) with washing buffer at 65°C. When the membrane had little radioactivity left, it was dried on filter paper keeping DNA/RNA side up and packed in a transparent foil. The signals were developed on Phospho imager screens and put in a cassette for up to a week. The screens were analysed using Typhoon scanner (Typhoon 9200 Variable Mode Imager) and its soft ware (Typhoon Scanner Control 3.0)

100 x Denhardt's: 2 % [w/v] BSA Fraktion V, 2 % [w/v] Ficoll 400, 2 % [v/w] PVP 360.000

Herings Sperm-DNA or Single Strand DNA (Biomol):

10 mg/ml dissolved in 1 x TE 2-4 h using magnet stirrer. Before making aliquots it was taken in and out of syringe many times and stored at -20 °C. Before using the DNA was boiled 5 min at 95 °C to denature.

Southern Blot Hybridizing Buffer: 15 ml 4 M NaCl, 10 ml 0.1 M Pipes, pH 6.8,
200 μ l 0.5 M EDTA, pH 8.5, 1 ml 10 % [w/v] SDS
10 ml 100 x Denhardt's, 63.7 ml Aqua dest.,
100 μ l denatured Herings sperm-DNA

Washing Buffer: 0.1 % [w/v] SDS, 2 x SSC

2.20 DNA extraction from *E.coli* at small scale (A quick and easy Miniprep method)

An easy and quick DNA extraction from *E. coli* was done according to Ahn *et al.*, 2000. The detailed protocol was as follows.

Single clone/colony was incubated overnight in approximately 3 ml LB + appropriate antibiotics at 37°C at 225-250 rpm. Next morning the bacteria were harvested by centrifugation in 1.5 ml eppendorf tubes at 11,000 g (13,200 rpm) for 1 min. The supernatant was removed and the obtained pellet was re-suspended completely in 100 µl re-suspension buffer. Then 100 µl of lysis buffer was added and mixed gently at room temperature. 120 µl neutralization buffer was added to the same aliquot, mixing was done by gently inverting the tubes few times and kept at room temperature for 3 minutes. The bacterial debris was removed by centrifugation at 11,000 g (or 13,200 rpm) for 1 minute at RT. Supernatant was then transferred to new eppendorf tube and 200 µl Isopropanol was added, mixed gently and kept at room temperature for 1 min to precipitate DNA. DNA pellet was collected by similarly centrifugation at RT. The supernatant was discarded and pellet washed with 500µl of 70% DNA. Pellet air dried for 3-5 minutes and dissolved in 20-25 µl sterilized milliQ water.

Re-suspension Buffer:	50mM Tris-HCl, 10mM EDTA pH 8.0, 20µg RNaseA
Lysis Buffer:	200mM NaOH, 1% SDS)
Neutralization Buffer:	3M Potassium Acetate pH 5.5

2.21 Maxi/Midi Prep for plasmid DNA

When high quality and higher amount of plasmid DNA is required then the kits for Midi and Maxi prep from Qiagen were used. The complete protocol was followed and only supplied reagents were used as recommended by the manufacturer.

2.22 Extraction of Plasmid-DNA from *Agrobacterium tumefaciens*

The agrobacteria containing required plasmid were cultured in 2 ml selective YEB liquid medium for about 21-24 h at 28°C on a shaker (225 to 250 rpm). Then pellets were

obtained by centrifugation (5 min, 6000 rpm, RT). The pellet is dissolved in 400 µl of buffer I and incubated for 10 min at RT. The alkaline lysis was done by adding 800 µl of buffer II. Solutions were mixed by gently inverting the tubes and kept for 10 min at RT. To separate eliminate, 120 µl buffer IIa was added, mixed gently in the same way and again neutralized with 600 µl 3 M Sodium acetate pH 5.2. The mixture was cooled for 15 min at -20 °C and centrifuged for 5 min at 14000 rpm and 4 °C. The clear supernatant was divided in 3 Aliquots; 650 µl each and in each aliquot 2 volume (1300µl) ice cold 100 % ethanol added. The mixture was again super cooled for 15 min at -80 °C for precipitation. The cooled mixture was centrifuged for 10 min at 14.000 rpm and 4°C. The obtained pellets were allowed to dry and again dissolved in a volume of 500 µl 0.3 M Sodium acetate, pH 7.0 and further 1000 µl 100 % ethanol was added to again precipitate DNA (15 min at -80°C). DNA Pellets were collected by centrifugation (10 min, 14.000 rpm, 4°C). The pellets were washed twice with 70 % ethanol, dried at RT and finally dissolved in 50 µl 10 mM Tris, pH 8.0. Contaminating RNA was removed by adding 3 µl of RNase A+T1 (Sigma) for 15 min. 5 to 9 µl was taken and run on 0.8 % agarose gel to analyse plasmid DNA.

Buffer I: 50 mM Glucose, 10 mM EDTA, 25 mM Tris, pH 8.0 4 mg/ml Lysozyme (added immediately before use)

Buffer II: 0.2 M NaOH, 1 % [w/v] SDS

Buffer IIa: 2 Volume of Buffer II, 1 Volume Phenol¹

2.23 Total RNA Extraction (Bartels *et al.*, 1990)

About 250 mg plant material was frozen with liquid nitrogen and ground to a fine powder in a pre-chilled mortal pastel. Plant material was then homogenized for about 30 seconds in 500 µl of pre-warmed (80°C) mixture of RNA extraction buffer and phenol in a 1:1 ratio.

RNA extraction buffer:

- 100 mM LiCl
- 100 mM Tris pH 8.0
- 10 mM EDTA pH 8.0
- 1% (w/v) SDS

This RNA extraction buffer was prepared, autoclaved and kept at bench (RT). Equal volume of phenol was added just before pre-warming.

This mixture was re-suspended in 250µl Chloroform: Isomylalcohol (24:1) for 30 sec. After this step the samples were kept on ice and other samples were homogenized in the same way. When all samples were homogenized, centrifugation was done at RT for 5 min and 14000 rpm. Supernatant was taken in new eppendorf tubes and equal volume of 4 M LiCl added and kept overnight in ice box and the boxed kept at 4°C. Centrifugation was then done for 20 minutes at 4°C and 14000 rpm in a pre-chilled centrifuge. The supernatant was carefully discarded and the pellet dissolved in 250µl sterile milliQ water. At this step, 0.1 volume (25µl) of 3 M sodium acetate pH 5.2 and 2 volumes (550µl) of absolute ethanol were added and this mixture was kept at -70°C for 2 hours. Again centrifugation was done at 14000 rpm and 4°C for 10 minutes. The supernatant was carefully discarded and the pellet was washed twice with 70% ethanol. For each washing, 1 ml 70% ethanol was added without dissolving the pellet and discarded after a centrifugation for 2 minutes. The pellet was air dried for a few minutes and re-suspended in 25µl RNase free water and stored at -70°C.

2.24 RNA transfer to Nylon Membrane (Northern Blot)

To transfer RNA for Northern-Blots denaturing agarose gel protocol was employed (Thomas, 1980). In this procedure formaldehyde plays an important role as it prevents the formation of secondary structures of RNA. At least 30 µg RNA was taken from each sample and mixed with the northern loading buffer in 1 to 1 ratio. After heating the samples at 70°C for 5 minutes, they were immediately loaded on a 1.5% northern agarose gel with formaldehyde.

Northern Electrophoresis Buffer

10x MEN	100 ml
dH ₂ O	820 ml
Deionised Formaldehyde (37%)	80 ml

10x MEN

200 mM MOPS (Mol. Wt.: 209.26)	41.8 g
80 mM Sodium Acetate pH 7.0	6.56 g
10 mM EDTA	20 ml of 0.5 M EDTA pH 8.0

dH ₂ O	upto 1 Litre
Northern Loading Buffer	
10 x MEN	50µl
Deionized Formaldehyde (37%)	175µl
Formamid	500µl
Bromo Phenol Blue (BPB)	0.2 mg or 20µl of 10% BPB solution
Glycerin	255µl of 100% Glycerin

Northern agarose gel (1.5 %)

2.25 (g) agarose taken in 108 ml distilled water, boiled to melt agarose and cooled down to 60°C. The mixture of 27 ml 37% formaldehyde and 15 ml of 10x MEN was added to and immediately poured onto the gel moulding tray. The electrophoresis was done at 100mA till the blue loading dye had moved at least 6 cm from wells. The blotting was done according to the standard capillary transfer using 20X SSC. RNA was bound with the membrane by exposing the membrane to UV for 2 to 3 minutes next morning or baked at 80°C for 1-2 hours.

2.24.1 Staining of the membrane with methylene blue:

Methylene blue solution

To check the efficiency of the transfer, the membrane was stained with a methylene blue solution [0.04% (w/v) methylene blue in 0.5 M sodium acetate, pH 5.2]. The membrane was immersed in methylene blue solution and put on shaker for 5-10 minutes at room temperature. The membrane was washed three times with water, each time gently shaking the membrane for few minutes. RNA and DNA bands stained blue against the white to bluish background of the membrane. The methylene blue stain detects > 20 ng RNA or DNA/band. The stain can be completely removed from the membrane by washing with 0.1-1 % SDS which is preset in a routine hybridization protocol and can be removed from the membrane with a pre-hybridization solution containing SDS.

2.24.2 Preparation of a $\alpha^{32}\text{P}$ -dCTP Radioactive-Labelled Probe (Feinberg and Vogelstein, 1983)

The radioactive labelling was done using Hexa LabelTM DNA Labelling Kit (Fermentas) according to the instructions of the manufacturer. The method provides a good alternative to nick translation for producing uniformly radioactive DNA of high specific activity. The method relies on priming of the polymerase reaction on the template DNA with random hexa-nucleotide primers. The complementary strand is synthesized from the 3'-end of the primer with the help of the Large Fragment of DNA Polymerase I, Exonuclease minus (Klenow Fragment, exo-) in the presence of labelled deoxyribonucleoside triphosphates (dCTPs). After the labelling reaction non-incorporated dNTPs were removed by chromatography on Sephadex G-50. For this 1 ml syringe was blocked with GF/C-Filter and filled with 1 ml Sephadex G-50 column in 1 x TE. The probe mix was passed through the column and 10 fractions of 100 μ l each of 1 x TE eluted. After measuring the counts of the fractions with the first peak was taken and denatured at 95°C for 5 minutes.

2.24.3 Hybridizing and Autoradiography

The membrane with nucleic acids was next incubated in northern hybridizing buffer for at least 2 hr at 42 °C in a water bath with shaker for pre-hybridizing. Then the buffer was renewed few minutes prior to the addition of denatured $\alpha^{32}\text{P}$ -dCTP radio labeled probe. The hybridization was done overnight. And the next day the hybridizing buffer was removed and the membrane washed to remove unspecifically bound probe. Washing was repeated 2 to 3 times (20 to 30 mins each time) with Washing Buffer at 42 °C. When the membrane had little Radioactivity left, it was dried on filter paper with the RNA side up and packed in a transparent foil. The signals were developed on Phospho imager screens and put in a cassette for up to a week. The screens were analysed using a Typhoon scanner (Typhoon 9200 Variable Mode Imager) and its soft ware (Typhoon Scanner Control 3.0)

Northern-Hybridizing Buffer: 50 ml 100% deionised Formamid, 25 ml 20 x SSC,
1 ml 100 x Denhardt's, 1 ml 0.1 M Pipes, pH 6.8,
500 μ l denatured Herings Sperm-DNA

Wash Buffer: 0.1 % (w/v) SDS, 2 x SSC

2.25 Protein extraction

Total proteins from leaves of plants were extracted according to Laemmli (1970). Briefly; adequate amount (50-100 mg) of plant material from fresh young tissues (leaves) were taken, frozen in liquid nitrogen and was grinded to a fine powder in a pre chilled mortal pestle. Equal amount (50-200 μ l) of Laemmli protein buffer was mixed and vortexed in the eppendorf tubes to homogenize it very well. The mix was baked on heat block at 95°C to 100°C for 5 minutes, cooled down on ice for a few minutes and then centrifuged at RT and 13000 rpm for 5 minutes. The supernatant was transferred into new tubes and was either run on SDS-PAGE or stored at -20°C. Before electrophoresis protein samples were again heated at 95°C for 2-3 minutes.

Laemmli Protein Buffer:	62.5mM	Tris HCl Ph 6.8
	10% (v/v)	Glycerin
	2% (w/v)	SDS
	0.7M	β -Mercaptoethanol
	0.1%	Bromo phenol blue (BPB)

2.25.1 Gel electrophoresis of Denatured Proteins (SDS-PAGE)

The protein samples were boiled for 5 minutes and then 20 μ g proteins/lane were loaded on to 12 % SDS poly acrylamide gel. The protein bands were separated on SDS-PAGE when current passed from cathode to anode at maximum of 20 mA for 1-1.15 mm thick gel till dark blue bands flows out. The gel was either processed for western blots or stained with coomassie brilliant blue till blue back ground was visible. Then the gel was de-stained till the bright blue bands of proteins were visible.

2 x SDS-Probe buffer:	62.5 mM Tris, pH 6.8, 10 % (v/v) glycerin, 2 % (w/v) SDS, 5 % (v/v) β -ME, 0.1 % (w/v) bromphenol blue
4 % Stacking gel:	3.05 ml aqua dest., 0.65 ml 30 % polyacrylamide or 0,8 % bisacrylamide (Roth), 1.25 ml 0.5 M Tris, pH 6.8, 50 μ l 10 % (w/v) SDS, 25 μ l 10 % (w/v) APS, 5 μ l TEMED
12 % Separating Gel:	4.95 ml aqua dest., 6 ml 30 % polyacrylamide or 0,8 % bisacrylamide (Roth), 3.75 ml 1 M Tris, pH 8.8, 150 μ l 10 % (w/v) SDS, 150 μ l 10 % (w/v) APS, 6 μ l TEMED

5 x SDS-Running Buffer: 15 g/l Tris, 72 g/l glycine, 5 g/l SDS

Coomassie-Staining Solution: 2.5 g/l coomassie brilliant blue R 250, 500 ml/l methanol,
70 ml/l glacial acetic acid

De-Staining Solution: 500 ml/l Methanol, 70 ml/l glacial acetic acid

2.25.2 Ponceau staining

Protein detection on nitrocellulose membrane was performed by ponceau staining [0.2 % (v/v) ponceau S in 3 % (w/v) TCA] after carrying out western blots and before antibody binding.

2.26 Purification of DNA Fragments from Agarose Gels

To get ALDH gene specific probes, after digestion with restriction endonucleases, PCR amplification of plasmid DNA constructs DNA bands or inserts for ectoine genes, agarose gels extraction and purification using QIAEX II extraction kit (Qiagen) or Macherey Nagel Nucleo Spin Extract II, per purification kit was employed. The extraction and purification was done after excising the required bands from agarose gel followed by the purification according to the instructions of the manufacturer (Qiagen or Macherey-Nagel).

2.27 Qualitative and Quantitative Estimation of Concentrations of Macromolecules

2.27.1 Qualitative and Quantitative Estimation of DNA and RNA

Extracted nucleic acids (DNA and RNA) were qualitatively compared after 1 % (w/v) agarose gel electrophoresis using 1 kb ladder. The concentration of the nucleic acids was spectrophotometrically determined at OD of 260 and 280 nm. A value of $OD_{260} = 1$ corresponds to 50 $\mu\text{g}/\mu\text{l}$ for DNA solution whereas $OD_{260} = 1$ corresponds to 40 $\mu\text{g}/\mu\text{l}$ for RNA solution. For a pure DNA extraction, the value of OD_{260}/OD_{280} must be between 1.8 and 2. A value of OD_{260}/OD_{280} below 1.8 means a contamination of DNA preparation with proteins or phenolic compounds. For the purity of RNA extraction, OD_{260}/OD_{280} value

must be higher than 2. A value of OD₂₆₀/OD₂₈₀ below 2 indicates a contamination of RNA extraction with proteins or phenolic compounds.

2.27.2 Quantitative Estimation of Protein Extracts

The estimation of protein concentration was carried out using a BioRad protein assay kit according to Bradford (1976). Protein aliquots (100 µl) were mixed with 200 µl BioRad protein assay kit and brought to 1000 µl with sterile H₂O (700 µl). The suspensions were incubated at room temperature for 5 min followed by an OD measurement at 595 nm. The quantification of the protein aliquots was calculated by calibrating the measurement of each sample with the OD (595 nm) against a given concentration (10 µg/µl) of standard proteins (BSA) curve.

2.28 Physiological and Biochemical Assays

2.28.1 Lipid Peroxidation Assay (MDA Analysis)

The plant material was ground with metal beads or in mortar and pestle and was transferred into 15 ml falcon tubes with 5 ml of chilled 0.1% (w/v) TCA solution. The mixture was vortexed and then incubated at RT for 5 min. This step was repeated twice and each time followed by centrifugation for 5 min at 4°C and 4000 rpm. The supernatant was transferred into new tubes; one aliquot of 0.6 ml the supernatant in 0.6 ml of 20% (w/v) TCA with 0.5% (w/v) TBA solution and another one with a 20% (w/v) TCA solution (without TBA) and incubated for 30 min at 100°C in a water bath. After cooling down to RT, the mixtures were centrifuged for 5 min at 13,000 rpm and RT. The absorbance of supernatant was measured at 440 nm, 532 nm and 600 nm.

The amount of MDA was calculated with the following formula (where FW= fresh weight):

$$\text{MDA equivalents (nmol/ml)} = [(\text{OD}_{532\text{TCA+TBA}} - \text{OD}_{600\text{TCA+TBA}}) - (\text{OD}_{532\text{TCA}} - \text{OD}_{600\text{TCA}}) / 157000] \times 10^6$$

$$\text{MDA equivalents (nmol/g fresh weight)} = 2 \times \text{MDA equivalents (nmol/ml)} \times \text{Total volume of the extracts (ml)} / \text{gram FW}$$

2.28.2 Determination of Chlorophyll Content (MacKinney, 1941 ; Arnon, 1949)

Total chlorophyll was extracted in 80% (v/v) aqueous acetone based on the work of MacKinney (1941) and spectrophotometrically quantified according to Arnon (1949). For the extraction, 0.2 g plant materials were used and suspended in 2 ml extraction buffer and incubated in dark under shaking at room temperature for 30 min. The suspension was centrifuged (5min, 10000 rpm, RT) and the OD of the supernatants was measured at 663 nm and 645 nm in quartz cuvettes. The chlorophyll content was estimated by the following formula as described by Arnon (1949):

$$C = 20.2 \times OD_{645} + 8.02 \times OD_{663}$$

where C expresses the total chlorophyll content (chlorophyll A + chlorophyll B) in mg/l of extraction solution.

2.28.3 Gas Exchange and Chlorophyll Florescence

Gas exchange measurements were done with the GFS 3000 (WALZ, Germany) at various light intensities. Using this apparatus chlorophyll florescence was also measured simultaneously from dark adapted tobacco plants.

2.28.4 Free Proline Content (Kant *et al.*, 2006; Deniz and Duzenli, 2007)

The proline content was measured according to Bates *et al* (1973). Plant material (40-50 mg) was ground in liquid N₂ using pre-chilled mortar and pestle and then homogenized in 1 ml of 3% sulphosalicylic acid. The mixture was centrifuged at 13200 rpm for 10 min. 0.5 ml of the supernatant or standard L-Proline solution was transferred into a 15 ml falcon tube and successively 0.5 ml of glacial acetic acid and 0.5 ml of ninhydrin acid were added. Solutions were mixed gently and the reaction mixture was incubated at 100°C in a water bath 60 min. The reaction mixture was cooled down to room temperature and then proline was extracted with 1 ml of toluene. The absorbance of the 0.7 ml toluene phase was read at 520 nm against toluene blank in cuvettes in quartz. The proline content was calculated using standard dilutions of L-proline.

Free proline content ($\mu\text{mol g}^{-1}\text{FW}$) = Estimated concentration x (L) volume of extract / FW

The proline content was calculated using standard dilutions of L-proline and expressed on an FW basis as follows

$$[(\mu\text{g proline/ml} \times \text{ml toluene}) / 115.5 \mu\text{g}/\mu\text{mole}]/[(\text{g sample})/5] = \mu\text{moles proline/ g of fresh weight material}$$

2.28.5 Electolyte Leakage percent

The electrolyte leakage of plants was measured according to Phillips *et al* (2008) with a conductivity meter (Qcond 2400) expressed as the percentage of conductivity measured in the water before and after boiling samples.

2.28.6 *In Vivo* Detection of H₂O₂ by the DAB-Uptake Method

For the localization of H₂O₂ generation as result of abiotic stress, leaf materials were taken from stress treated and unstressed plants and placed in 1mg/ml 3,3'-diaminobenzidine (DAB)-HCl, pH 3.8 (Sigma, a low pH is necessary in order to solubilize DAB) and adjust the pH to 7.5 after solubilization. Samples were incubated in a growth chamber for 8 h and cleared by boiling the leaves in 80 % (v/v) ethanol for 2 h and finally imbedded in 10 % (v/v) glycerol. The accumulation of H₂O₂ was observed as brownish stains in the leaves (Thordal-Christensen *et al.*, 1997).

2.28.7 H₂O₂ Quantification

A modified method of Rao *et al* (2000) was used for H₂O₂ measurement. Briefly, 200 mg plant material was treated with the DAB up-take method as described above, followed by chlorophyll clearing by boiling the sample in 96 % (v/v) ethanol (20 min) and immediately homogenized in 1 ml of 0.2 M HClO₄ in a pre-cooled pestle and mortar. The mixtures were incubated on ice for 5 min and centrifuge (10,000 g, 10 min, 4°C). The optical density (OD₄₅₀) of the supernatants was measured as described by Tiedemann (1997) and the H₂O₂ concentrations were obtained using standard solutions of 0.2 M HClO₄, containing 5, 10, 25, 50 μMol H₂O₂ (Sigma). These standards were used to calibrate the data at the same OD (450 nm) during each assay run.

2.29 Cloning of DNA fragments

2.29.1 Primers designing for cloning

For PCR amplification, DNA sequencing and various plasmid DNA constructs, specific primers were designed with the following criteria:

The GC content of a primer must be approximately 50%, and the melting temperature (T_m) should be according to Faust rules $T_m = 4 (G+C) + 2 (A + T)$ where G, C, A, T represent the DNA bases of the primer sequence. T_m should be approximately between 60 to 65°C. Based on the fact that GC are complemented (linked) with three hydrogen bonds, the primers preferentially ended with at least one dGTP or dCTP. The primers were designed to avoid self-complementation forming thereby a secondary structure. Forward and reverse primers of each PCR reaction were designed to have approximately the same T_m.

The primers were designed to contain at least at their 5'-end a minimum of specific number of base pairs required for the corresponding restriction site in order to ensure a possible and subsequent digestion of the amplified DNA fragments by the restriction endonucleases.

2.29.2 Polymerase chain reaction (PCR)

DNA fragments were amplified from various plasmid DNA constructs or genomic DNA as described below:

For each PCR reaction a total volume of 50 µl solution was prepared as followed:

30-35 µl	sd H ₂ O
5.0 µl	10 x PCR-buffer (GibcoBRL/Invitrogen)
1.5 µl 50	mM MgCl ₂ (GibcoBRL/Invitrogen)
2.0 µl	Fwd-primer (10 pmol/µl)
2.0 µl	Rev-primer (10 pmol/ µl)
1.0 µl	10 mM dNTPs
1.2 µl	plasmid DNA (5 ng/ µl) or PCR product (5 ng/ µl) or bacterial clones (tooth picks or yellow tips dips), or 1 to 5 µl of genomic DNA solution
1.5 µl	1:10 diluted Taq-polymerase (Pluthero, 1993) into Taq-buffer

Taq-buffer: 50 mM Tris, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF,

50 % (v/v) glycerol, pH 7.9, filter sterilized and stored at -20°C.

Each PCR aliquot (50 µl) was mixed. PCR reactions were performed in a TRIO thermo block (Biometra, Göttingen). The optimal number of PCR cycles and the annealing temperature was determined empirically per PCR reaction. A standard PCR programme was as followed:

94 °C	3 min of initial denaturing	
94 °C	30 sec of denaturing	} 35 cycles
T _A	30 sec of primer binding	
72 °C	30 sec of DNA synthesis	
72 °C	5 min at the end of the reaction	
4 °C	indicating the end of PCR running programme	

T_A = annealing temperature = T_m – 4 °C

T_m = melting temperature

2.29.3 Restriction Endonuclease Treatments

DNA digestion was carried out by restriction endonucleases according to the following criteria: the reaction buffer (10x) was 1/10 of the end volume and 2-5 U of restriction enzymes was used per 1 µg of DNA to be digested. A double digestion was possible per reaction only when both restriction enzymes used the same buffer otherwise the digestions were performed chronologically.

2.29.4 Dephosphorylation

Digested DNA fragments were dephosphorylized at their 5' end with shrimp alkaline phosphatase (SAP, Boehringer/Roche, Mannheim, Germany) in order to avoid re-ligation of cohesive-ends of plasmid DNA during DNA recombination. The reaction was made in 5 µl (end volume) comprising of 0.5 µl dephosphorylation buffer (10 x), 1.0 µl (1 unit) SAP and appropriate quantity of plasmid DNA. The mixture was made up to 5 µl with sterile

distilled water. The solution was incubated for 10-20 min at 37 °C and followed by heat inactivation of SAP at 65 °C for 15-20 min.

2.29.5 Ligation

For plasmid DNA constructs, ectoine genes DNAs inserts were ligated in various independent steps. The ligation reaction was brought to 10 µl (end volume), which comprises 1.0 µl ligase buffer (10x), x µl (50 ng) digested plasmid DNA vector (with or without dephosphorylation), 1.0 µl T₄ DNA ligase (Roche), and y µl (150 ng) DNA insert. The mixture was adjusted to 10 µl with sterile H₂O and incubated at 16°C for 20 h. For a good ligation reaction the ratio of plasmid vector to that of the DNA insert in the mixture was at least 1 to 3.

2.30 Transformation

2.30.1 Preparation of Chemically-Competent *E. coli*

Single colony from freshly streaked LB Agar plates with *E. coli* strain DH10B was cultured in 5 ml LB for overnight (37°C and 200 rpm). Next morning 2x2ml of this pre-culture is used to incubate 2x200 ml LB and was allowed to grow (37°C, 250 rpm) till OD₆₀₀=0.5, cooled in ice for 5 min and centrifuged (5 min, 5000 rpm, 4°C). The pellet was suspended in 1 ml cold 0.1 M CaCl₂ and further centrifuged as above. The pellet was resuspended in 9.0 ml cold 0.1 M CaCl₂ and centrifuged again as above. The pellet was finally resuspended in 1 ml 0.1 M CaCl₂, 15 % (v/v) glycerol and stored at -70°C in aliquots of 100 µl of competent cells.

2.30.2 Preparation of Electro-Competent Cells of *E. coli*

Single colony of *E. coli* strain DH10B was picked from freshly streaked LB Agar plates and cultured in 10 ml LB and cultured for overnight (37°C and 200 rpm). Next morning 2x2ml of this pre-culture is used to incubate 2x200 ml LB. Let them grow for almost 3 to 4 hours till OD₆₀₀ reached 0.6. The cells were kept on ice for 30 minutes (8x 50 ml falcon tubes). Centrifugation was done for 5 min at 4°C and 5000 rpm. The cells were washed with 50 ml Aqua distilled (ice cold). Similarly centrifugation was done. Cells were re-suspended in 25 ml sterile distilled water (ice cold). Centrifuged similarly and the all pellets were put in 2 tubes (4 in each). The pellets were washed twice with 25 ml 10%

(v/v) glycerine (ice cold) and re-suspended in 5 ml GYT media (ice cold). Similarly centrifuged and finally the pellets were re-suspended in 1 ml GYT (ice cold). 50 μ l aliquots were made, immediately frozen with liquid Nitrogen and stored at -70°C.

GYT Media: 10% (v/v) Glycerine
 0.125% (w/v) Select Yeast Extract
 0.25% (w/v) Tryptone

2.30.3 Preparation of Electro-Competent *A. tumefaciens*

A single colony of agrobacterium (GV3101 or LBA4404) was picked from freshly streaked plate and pre-cultured in 3 ml YEB_{Rif} medium and incubated in a shaker (225 to 250 rpm) for 14-16 hours at 28°C. The agrobacterium culture from this pre-culture was poured in fresh 50 ml YEB_{Rif} medium and incubate similarly for about 8 hours till the OD₆₀₀=0.5. The bacterial cells were incubated for 30 min on ice (4°C). The agrobacterium cells were then passed through a series of centrifugations each time 5.000 rpm, 10 min, 4 °C and then again resuspended in the subsequent solutions in the following manner:

25 ml 1 mM Hepes, pH 7.5
12.5 ml 1 mM Hepes, pH 7.5
10 ml 10 % (v/v) Glycerine, 1 mM Hepes, pH 7.5
5 ml 10 % (v/v) Glycerine, 1 mM Hepes, pH 7.5
2 ml 10 % (v/v) Glycerine

The pellet was finally dissolved in 1 ml 10 % (v/v) Glycerine. 40 μ l Aliquots were made and immediately frozen with the help of liquid nitrogen and then stored at -70 °C.

2.30.4 Transformation of Chemically-Competent *E. coli*

1-2 μ l plasmid DNA (5 -50 ng/ μ l) or ligated plasmid DNA construct was added to one aliquot of calcium-competent cells (100 μ l) and carefully mixed. The mixture was incubated in ice for half an hour and then heat shock was given in a water bath at 42 °C for 90 sec and cooled again on ice for few minutes. LB medium (650 μ l) was added to the transformed cells and further incubated (37 °C, 250 rpm) for 1 h for antibiotic recovery. Before plating, 1:10 and 1:100 dilutions of the transformed cells were made with LB

medium. Aliquots (100-200 μ l) of the diluted cells were spread on LB agar selective plates and incubated at 37°C overnight.

2.30.5 Transformation via electro-poration (Tung and Chow 1995)

Aliquots of electro-competent cells were thawed on ice before transformation. About 1-2 μ l DNA of a ligated vector or specific plasmid DNA (approximately 5-10 ng/ μ l) was added to the competent cells and carefully mixed in a pre-cooled electro-poration cuvette and passed through 3 to 5 sec electric shock (GenePulser II, BIO-RAD) for the transformation. 1 ml YEB-medium or 800 μ l SOC was added to the transformed cells and further incubated in a glass tube for another 1 h at 37°C (for *E. coli* transformation) or 3 h at 28°C (for *A. tumefaciens* transformation) shaking at 250 rpm. 100 μ l aliquots of the cells were finally plated out overnight with the selected antibiotics at appropriate temperatures as indicated as follows

	<i>E. coli</i> :	<i>A. tumefaciens</i> :
DNA to be transformed	Ligation mix or Plasmid	Plasmid
Electroporation cuvette (Bio-Rad)	1 mm	2 mm
Capacitance	25 μ F	25 μ F
Volts	1.6 kV	2.5 kV
Resistance	200 Ω	400 Ω
Liquid Medium after electroporation	SOC/LB	YEB
Incubation temperature	37°C	28 °C
Time: Till development of colonies	12-16 h	48-72 h

2.31 Agrobacterium Mediated Stable Transformation of Tobacco (*Nicotiana tabaccum*) Wild Type SR1 Plants

2.31.1 Preparing Agrobacterium

10 ml YEB with antibiotics (Rifampicin 100 μ g/ml and Spectinomycin 100 μ g/ml) was inoculated with the single colony of agrobacterium containing the desired clone. This culture was incubated for 2 days on shaker (250 rpm) at 28°C in dark. The culture was transferred to 100 ml YEB+ antibiotics and incubated over night on shaker (250 rpm) at

28°C in dark till the OD₆₀₀ reached about 1.0. Bacterial cells were harvested by centrifugation at 6000 rpm, 4°C, and 10 min in 250 ml Beckmann tubes. This was done to get rid of YEB media from the *Agrobacterium* cells so that plant material may not be harmed with YEB, as sometimes the excess YEB results in overgrowth of *Agrobacterium* colonies. Using 10 ml pipette, the pellet was carefully resuspended in 40 ml (2x20 ml) pre-cooled 10 mM MgSO₄. Again, it was centrifuged at 5300 rpm, 4°C, 10 mins in 50 ml Falcon tubes. Finally the pellet was carefully resuspended in 45 ml pre-cooled MS I (MS-H + Glucose) liquid. This final solution was used for transformation without any dilution.

2.31.2 Tobacco Transformation

Leaf discs of 1 cm² were cut from young leaves of sterile cultured tobacco plants. For bigger leaves (not too old) the mid rib was cut away and finally the leaf was cut into 4 pieces and left in ½ MS-H + Glucose medium until all leaf discs were prepared. The bacterial suspension was distributed to 3 Petri dishes (15 ml in each). Leaf discs were put upside down onto the suspension (without immersing them in the bacterial suspension) for about an hour and a sheet of aluminium foil was put over the petri dishes to keep the bacteria in darkness because they are light sensitive. After an hour the leaf discs were put upside down on MS I with Agar (6-10 pieces per plate) without washing. Plates were sealed with para film and incubated in culture room in darkness (Stack of plates were wrapped in Aluminium foil)

2.31.3 Selection for Transformed Cells and Development of Transformed Calli and Plants

The leaf pieces were washed 3 to 4 times (5 min each) in MS-H glucose + Cefataxime (500mg/ml). For this about 25 to 30 pieces were put in a 50 ml Falcon tube, filled with 50 ml MS-H glucose + Cefataxime and inverted carefully for 5 minutes. Then the leaves were dried on a sterile filter paper for few minutes and cultured upside down on MS II plates. The plates were put in culture room at 25°C with 16 hours light. The first subculture was done after 1 week to fresh MS II plates, later on every 2-4 weeks sub culture to fresh MS II plates was done. After shoots had developed (at least 1 cm) they were cut and transferred to MS III medium in magenta boxes. The roots developed from plants in 3 to 4 weeks and as soon as the roots had developed the plants were transferred into pots containing soil mix and vermiculite (4:1) and acclimatized in growth chamber.

Media:

Stocks

Vitamins:	Glycine	2 mg/ml	} dH ₂ O (Sterile filtered, stored at 4°C)
	Niacin (Nicotinic acid)	0.5 mg/ml	
	Pyridoxine. HCl	0.5 mg/ml	
	Thiamine. HCl	0.1 mg/ml	
	Claforan (Cefotaxime)	100 mg/ml	}
	BAP (Benzylaminopurine)	1 mg/ml	
	NAA (Naphtylacetic acid)	1 mg/ml	
	(BAP + NAA have to be presolved in 1	M NaOH)	
	Kanamycin: 50 mg/ml	dH ₂ O (Sterile filtered, stored at -20 °C)	

Washing Medium

MS-H+glucose + 500 mg/l Cefotaxime/clofaran

MS I (= MS-H+glucose with agarose)

MS-salts (micro- und macroelements, DUCHEFA) 4.496 g/l

Inosit (myo-Inositol) 100 mg/l

Glucose (Glucose monohydrate) 30 g/l

Agarose 8 g/l

pH 5.8

autoclaved

MS Vitamin-stock dH₂O (Sterile filtered, stored at 4°C) 1 ml/l

MS II

MS I +

Claforan-stock 5ml/l (= 500 mg/l)

Kanamycin-stock 1 ml/l (= 50 mg/l)

BAP-stock 500 µl/l (= 0.5 mg/l)

NAA-stock	100 µl/l (= 0.1 mg/l)
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MS III

MS-salts (micro- und macroelements, DUCHEFA) 4.496 g/l

Inosit (myo-Inositol)	100 mg/l
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Sucrose	20 g/l
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Agarose	8 g/l
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pH 5.8

autoclaved

Vitamin-stock	1 ml/l
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Claforan-stock	5ml/l (= 500 mg/l)
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Kanamycin-stock	2ml/l (= 100 mg/l)
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2.32 Stable Transformation of Tomato (*Lycopersicon esculentum* cv Money Maker)

2.32.1 Seed sterilization and germination

After seeds sterilization about 25 seeds were cultured in each glass jars (Magenta boxes) containing 100 ml of MS medium. The seeds were kept for 2-3 days in the darkness at 25°C. For this purpose, aluminium foil was wrapped around the glass jars. Later, they were transferred to growth room at 16hr/8hr light and dark period and 23°C and 18°C just few days before transformation.

2.32.2 Pre-Culture

After 8-10 days of culture the cotyledons were cut under sterile bench. The chosen cotyledons were about 1 cm long and regularly grown, rolled and irregular shaped cotyledons were discarded. At this stage the first true leaves not yet developed on seedlings. Cotyledons were cut off from the seedling, also from top and bottom edges and a longitudinal cut was made to proximal (lower) side. The cut-cotyledons were cultured on MS-B for one day.

2.32.3 *Agrobacterium* preparation

Agrobacterium clone containing the desired construct were streaked on freshly prepared selective YEB agar plates from glycerol stock 2-3 days before needed. Single colony from the plate was picked and the cells were cultured in tubes containing 6 ml of liquid YEB medium with appropriate antibiotics and incubated at 28°C overnight with shaking (approximately 200 rpm). 100µl to 200 µl from this starter culture was taken and diluted into fresh 250 ml liquid YEB medium again with appropriate antibiotics. Overnight cultures diluted by liquid MSO 2% to the appropriate concentration (OD₆₀₀=0.5). Bacterial cells were harvested by centrifugation at 6000 rpm, 4°C, and 10 min in 250 ml Beckmann tubes. This was done to get rid of YEB media from *Agrobacterium* cells so that plant material may not be harmed with YEB, because sometimes the excess YEB results in overgrowth of *Agrobacterium*. Using 10 ml pipette the pellet was carefully re-suspended in 40 ml (2x20 ml) pre-cooled 10 mM MgSO₄. Again it was centrifuged at 5300 rpm, 4°C, 10 mins in 50 ml Falcon tubes. Finally the pellet was carefully re-suspended in 45 ml pre-cooled MSO 2% and this suspension was used for transformation without any dilution.

<u>MSO 2%</u>	MS Salts (Duchefa)	4.3 g
	Myo-Inositol	100 mg
	Thiamine HCl (1mg/ml)	0.4 ml
	Glucose	20 g

Media Composition for Each Litre

	Seed Germination MS (MS-A)	Preculture and Co- cultivation (MS-B)	Callus and Shoot Regeneration (MS-C)	Shoot Elongation (MS-D)	Rooting media (MS-E)
MS Salts	4.3 g	4.3 g	4.3 g	4.3 g	4.3 g
Sucrose	15 g	30 g	---	---	15 g
Glucose	---	---	30 g	15 g	---
Vitamin (1000X)	1 ml	1 ml	1 ml	1 ml	1 ml
NAA stock	---	2 ml	---	---	---
BAP stock	---	2 ml	---	---	---
Zeatin	---	---	2 mg to 1 mg	1 mg	---

Riboside					
IBA	---	---	---	---	2 mg
GA	---	---	---	1 mg	---
Kanamycin	---	---	100 mg	100 mg	100 mg
Cefatoxime	---	---	500 mg	150 mg	75 mg
Agar	8 g	9 g	8 g	8 g	8 g

About 8 to 10 ml of the liquid culture containing agrobacterium was poured in the petri plates for 30 minutes with occasional swirling. The agrobacterium suspension was aspirated from the plates very well with the help of micro-pipette and the cotyledons were co-cultivated with the left over agrobacterium for about 2 days in darkness at 27°C.

2.32.4 Regeneration

After 48 hours of co-cultivation, the cotyledons were transferred to petri plates with MS-C medium keeping their adaxial side (upside) up. First subculture was performed after one week and later on the cotyledons were sub-cultured to fresh MS-C medium every 2 weeks. Callus with shoot primordia formed after 4-5 weeks. The callus was cut into pieces and piece with shoot primordium was sub cultured to MS-D medium in magenta jars for shoot elongation. Only one shoot (1 cm) was taken from each callus and rest of callus was discarded to make sure that each shoot remain an independent transforming line. If the transformation had at least 5-10 independent transformants it was taken as successful transformation. When shoot reached 4-5 cm it was excised from callus and sub-cultured on MS-E in magenta jars for rooting. Transformed developed a good root system and elongated further 2-3 weeks after transferring to medium MS-E. If not, cut stem was re-cut and transferred to fresh medium. Plantlets of about 5 cm were transplanted to pots containing soil mix and vermiculite (4:1). The pots were covered with a transparent plastic for few days and gradually acclimatized.

2.33 *Agrobacterium* mediated transformation of *Arabidopsis* plants (Floral Dip)

Wild-type plants were transformed via *Agrobacterium tumefaciens* mediated transformation with the ectoine cDNA constructs under the control of various promoters using a modified method of Clough and Bent (1998). The *A. thaliana* wild type (Col 0)

seedlings were allowed to grow to the stage of inflorescence. The *Agrobacterium* clone carrying the transgene was incubated (28 °C, 250 rpm) in 250 ml selective YEB (Spectinomycine 100mg/l and Rifampycin 50mg/l) till OD₆₀₀ of the culture reached 0.9 and centrifuged (5000 rpm, 10 min, 4 °C) in a 250 ml centrifuged bottles. The pellets were resuspended in a minimum volume of 400 ml infiltration medium [0.01% (v/v) Silwet 77 in YEB] and further incubated using a stirring magnet till OD₆₀₀ = 0.8. The infiltration medium was prepared in a 500 ml beaker to enable the wild-type seedlings to be immersed in the solution for transformation. The pots containing the wild-type *Arabidopsis* plants were carefully inverted and immersed in the infiltration medium while stirring for 1 min. Care was taken to submerge all the earlier inflorescence of the seedlings in the solution. The seedlings were thereafter placed back in the trays and sealed in plastic bags. Few holes were made in the bags for aeration. Three days after infiltration the plastic bags were removed and the seedlings were supported together with a stick and allowed to grow till the first generation of seeds (T1).

2.33.1 Screening for positive transformed clones

Using a replica plating technique, cell clones were transferred into new plates and assigned appropriate numbers, which were considered throughout the screening process. The clones were either used as DNA source to amplify DNA inserts via PCR amplification or used for individual plasmid mini prep from which appropriate DNA digestions were carried out using specific restriction enzymes in order to check the DNA fragments and confirm the correct insertion.

2.33.2 Screening for transgenic *Arabidopsis* seeds

After transformation, the first generation of seeds (T1) was collected, surface sterilized and sown on MS-agar plates containing appropriate selection marker (50 mg/l Kanamycin final concentration). After 15-days of growth, transgenic seeds were able to germinate and produce green leaves in the presence of the selective marker while the non-transgenic seeds were not able to grow with true and healthy green leaves. The positive seedlings (transgenic lines) were transferred into soil trays and allowed to grow for the next generation of seeds (T2)

2.34 Soluble Osmolyte Ectoine

As the second part of studies additional experiments were planned with the group of Prof. Galinski (Institute of Microbiology & Biotechnology, Uni-Bonn) and the three genes responsible for the synthesis of soluble osmolyte (Ectoine) were obtained from Prof Galinski, that have been isolated from the halophilic bacterium *Marinococcus halophilus*, amplified with PCR, digested with restriction enzymes and respective restriction sites created into three separate vectors (Chung *et al.* 2005) so that each genes is with a different promoter and terminator (Cassette). This is done to avoid any gene silencing when more than one gene is in same vector and transformed into plants via *Agrobacterium*.

The idea is that all three genes encoding the enzymes for the ectoine biosynthesis should be cloned onto the same plant transformation vector.

2.35 Cloning Strategy of Soluble Osmolyte Ectoine Genes

2.35.1 Co-bombardment of Ect A, Ect B and Ect C genes

Gold micro-carriers were prepared for 120 bombardments using 600 µg of the micro-carrier per bombardment, based on the method of Sanfor *et al.* (1993). For this 30 mg gold micro-particles were weighted in 1.5 ml microfuge tube. 1 ml of HPLC grade 70% ethanol (v/v) is added. Put on plat-form vortexer for 3-5 minutes. The particles were allowed to soak in 70% ethanol for 15 minutes then a brief (3-5 sec) centrifugation was done to pellet the micro-particles. The supernatant was discarded. The following wash steps were repeated thrice

- Addition of 1 ml of sterile water
- Vortexed vigorously for 1 minute
- The particles were allowed to settle down for 1 minute
- Spinning down in microfuge for short time
- Supernatant was discarded.

After three washes, 500µl of sterile 50% glycerol was added to make the final concentration of the micro-particle to 60mg/ml (based in the assumption that there is no loss during preparation). The aliquots with gold micro-carriers were stored at 4°C.

Coating Micro-carriers with DNA

For each five bombardments the following procedure was used:

The stored micro-carriers in 50% glycerol were vortexed for 5 minutes in a platform shaker to re-suspend and disrupt agglomerated particles. 50 μ l (3mg) of the microcarriers were taken into a 1.5 ml microcentrifuge tube. The DNA of the binary vector and the three primary vectors were taken in a ratio of 1:2:2:2 (Chen *et al.*, 1998) and added to the tube on platform shaker. Then 50 μ l 2.5M CaCl₂ and 20 μ l 0.1M spermidine (free base, tissue culture grade) were added into the same tube. The tube is left on the plat-form shaker for about 10 minutes. Then the microfuge tube put at microfuge stand and the particles were allowed to settle down for 1 minute. The supernatant was discarded and 140 μ l of 70% ethanol (HPLC grade) added. The tube was again vortexed briefly for few seconds only and the supernatant discarded again. Then 100% ethanol (HPLC grade) was added and the mixture vortexed briefly. The supernatant was discarded away and finally the micro-carriers pellet gently resuspended in 50 μ l of 100% ethanol. The micro-carriers were bombarded with the gene gun (Bio-Rad) according to the manufacturer's instruction on various tissues of tomato. The medium was changed after 3 days to MS medium with Kanamycine (100 μ g/ml) to avoid the callus development from non transformed tissues.

2.35.2 Cloning with Point Mutagenesis and PCR Amplification and Agrobacterium mediated transformation

- 1) PCR amplification of Ect B gene from pos M11 using *Pfu* polymerase creating NcoI and BamHI recognition sites at its ends. After digestion with restriction enzymes and phenol chloroform washing the PCR product was ligated into pSAT6.rbcP.MCS.rbcT (DQ 005473) that was also digested and purified in the same way.
- 2) PCR amplification of the portion with Ect B gene and the promoter and the terminator creating PacI recognition sites at its both ends using *Pfu* polymerase. Insertion of the PCR product directly into pJET1.2/blunt cloning vector (Fermentas). Digestion of the right clone with PacI restriction enzyme and its ligation with the binary vector (pRCS2-ocs-nptII or DQ005456) that was also digested with PacI and then dephosphorylated.

- 3) PCR amplification of Ect C gene from pos M11 using *Pfu* polymerase creating NcoI and Hind III recognition sites at its ends. After digestion with restriction enzymes and phenol chloroform washing the PCR product was inserted into pSAT7.actP.MCS.agsT (DQ 005453) that was also digested and purified in the same way.
- 4) PCR amplification of the portion with Ect C gene and the promoter and the terminator creating AflII recognition sites at its both ends using *Pfu* polymerase. Insertion of the PCR product directly into pJET1.2/blunt cloning vector (Fermentas). Digestion of the right clone with AflII restriction enzyme and its ligation with the binary vector (pRCS2-ocs-nptII or DQ005456) additionally containing Ect B gene and its promoter terminator (or the Ect B cassette) that was also digested with AflII and then dephosphorylated.
- 5) PCR amplification of Ect A gene from pos M11 using *Pfu* polymerase creating NcoI and SalI recognition sites at its ends. After digestion with restriction enzymes and phenol chloroform washing the PCR product was inserted into pSAT4.35SP.MCS.35ST (DQ 005466) that was also digested and purified in the same way.
- 6) PCR amplification of the portion with Ect A gene and the promoter and the terminator creating SbfI recognition sites at its both ends using *Pfu* polymerase. Insertion of the PCR product directly into pJET1.2/blunt cloning vector (Fermentas). Digestion of the right clone with SbfI restriction enzyme and its ligation with the binary vector (pRCS2-ocs-nptII or DQ005456) additionally containing Ect B gene cassette and Ect C gene cassette that was also digested with SbfI and then dephosphorylated.

After every insertion/ligation the DNA was transformed into electro-competent *E. coli* strain DH10B. Before proceeding to next step the right clone was identified with restriction digestion/colony PCR and to confirm further sequencing was done with the specific primers. The final construct or binary vector containing all the three ectoine genes and the promoter and terminators were used to transform electro competent *Agrobacterium* strains LBA 4404 and GV3101. Before using the *Agrobacterium* clones for plant transformation colony PCRs were done to confirm the presence of all the three genes.

2.36 Stress Treatments for *Arabidopsis thaliana* Over-Expressing ALDH Genes

2.36.1 Drought Stress

After the seeds were selected on MS + Kanamycine medium, seedlings were allowed to grow further 2 weeks on a soil mix in pots at $25 \pm 2^{\circ}\text{C}$ with 16 hr light. When the plants grew to a suitable size DNA was extracted and PCR was done to check the presence of the two ALDH genes, ALDH3I1 and ALDH7B4. Plants containing both genes were put under drought stress (water was withheld) for two weeks. The control plants were watered regularly.

2.36.2 High Salt or Salinity Stress

The seeds were selected on MS + Kanamycine medium for 2 weeks and then seedlings were transferred to soil mix in pots and allowed to grow further 2 weeks at $25 \pm 2^{\circ}\text{C}$ with 16 hr light. When the plants grew to a suitable size DNA was extracted and PCR was done to check the presence of the two ALDH genes, ALDH3I1 and ALDH7B4. Plants containing both genes were put under high salt (200mM NaCl) for two weeks. Every pot was watered with 50ml of 200mM NaCl solution every alternate day. The control plants were watered with same amount of tap water.

2.36.3 *In vitro* Salt Treatment

Wild type seeds were cultured in MS medium while transgenic lines were cultured of MS + Kanamycin (50mg/l) to screen out non-transgenic seeds. Seedlings were allowed to grow for about 2 weeks (till rosette stage) and then transferred to MS medium + 150 mM NaCl. At the end of the treatment, plants were weighed and one small leaf from each plant is taken to do PCR for the confirmation of the ALDH genes presence. Only positive plants were subjected to further analysis of MDA or chlorophyll content measurements.

2.37 Stress Treatments for ALDH Over-Expressing Tobacco (*Nicotiana tobaccum*)

2.37.1 Paraquat Induced Oxidative Stress (Rodrigues *et al.*, 2006)

For paraquat induced oxidative stress, 12 mm leaf discs from 2-month-old transgenic and untransformed plants were incubated with a series of paraquat (methyl viologen, Sigma) concentrations (0, 2, 5, and 10 μM) and kept for 1 hour in the dark, followed by incubation for 24 hours with a light intensity of 200 $\mu\text{mol m}^{-2}\text{s}^{-1}$ at 24°C. The extent of the oxidative damage was assessed by H_2O_2 in vivo detection by DAB uptake method (Thordal-Christensen, 1997) and H_2O_2 quantification (Rao *et al.*, 2000). Moreover, MDA and chlorophyll contents were also measured according to standard protocols.

2.37.2 Freeze Tolerance (Li *et al.*, 2007)

Both transgenic and wild type plants were potted in a growth room at 23°C. Freezing stress will be performed according to Konstantinova *et al.* (2002) with slight modifications. Plants at 5–6 weeks age were transferred to cold-acclimation at 4°C for 1 week under 14 hrs light (25 $\mu\text{mol m}^{-2}\text{s}^{-1}$)/10 hrs dark. Samples were collected before cold acclimation (the untreated) and after cold stress (the treated); the untreated plants were grown in parallel at 23°C so that they will be equal in age to the treated plants. The electrolyte leakage, gas exchange, MDA levels and chlorophyll content were measured.

2.37.3 Water Stress (drought) Treatments (Rodrigues *et al.*, 2006)

The water-stress treatment (drought) was induced in soil-grown tobacco plants, as described by Alvim *et al.* (2001). Briefly, transgenic T₂ seeds were germinated in kanamycin-containing medium for 3 weeks before transplantation. Plants were grown in a mixture of soil, sand, and dung (3:1:1 by vol.) for 2 weeks in the greenhouse under natural conditions of light, relative humidity at 70% and controlled temperature, 18°C and 25°C (night and day). After 30 d of growth with normal water supply, drought stress was imposed by withholding water for 2 weeks from half of the transgenic plants. The remaining transgenic plants received normal water supply continuously. In control experiments, the same conditions of water availability were applied in untransformed control plants at the same developmental stage as transgenic plants. The electrolyte leakage, gas exchange, MDA levels and chlorophyll content were measured.

2.37.4 High Salt or Salinity Stress

The seeds were selected on MS + Kanamycin medium for 2 weeks and then seedlings were transferred to MS and MS + 200mM NaCl and allowed to grow further 3 weeks at $25 \pm 2^{\circ}\text{C}$ with 16 hrs light.

3 Results

3.1 *Arabidopsis thaliana* ALDH genes

3.1.1 Preliminary experiments on *Arabidopsis thaliana*

Preliminary experiments on *A. thaliana* ALDH double over-expressing lines lead us to screen out 14 lines. Out of 14 lines four best performing lines were selected for further experimentation and characterization. Screening was performed on *in vitro* MS + salt (150 mM and 250 mM NaCl) and paraquat (0.5 μ M and 1.0 μ M). Leaf chlorosis scoring showed a higher chlorosis of leaves in wild-type but surprisingly some double over-expressing lines (SA37P7, SA37P9 and SA37P12) also showed a high leaf chlorosis (Fig 3.1) under 250 mM NaCl in the medium. However, salt level of 150 mM in MS medium gave a better picture of salt tolerant lines. Wild-type plants were most affected by 150 mM NaCl in the medium; causing very high chlorosis. Surprisingly a double over-expressing line SA37P11 performed even worse than wild-type. Among double over-expressing lines SA37P6, SA37P10, SA37P13 and SA37P44 were the most tolerant lines.

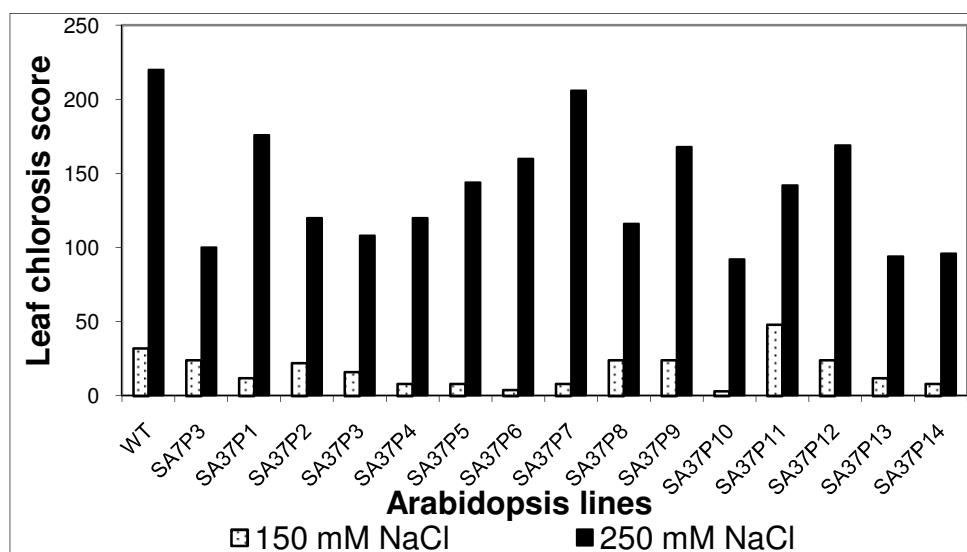


Fig. 3.1: Leaf scoring on the basis of loss of green colour under *in vitro* salt stress in ALDH3I1 and ALDH7B4 double mutants of *A. thaliana*.

When paraquat is added in the MS medium it serves as oxidative stress producing agent to plants growing on it. Wild-type plants failed to grow properly on either concentration of paraquat (Fig. 3.2) single over-expressing lines of ALDH3I1 and ALDH7B4 also could not execute normal growth and had higher leaf chlorosis than double over-expressing lines. However, double over-expressing line SA37P2 was the worst and almost all leaves turned yellow on 1.0 μ M paraquat medium. The best performing lines appeared to be SA37P6, SA37P10, SA37P13 and SA37P44.

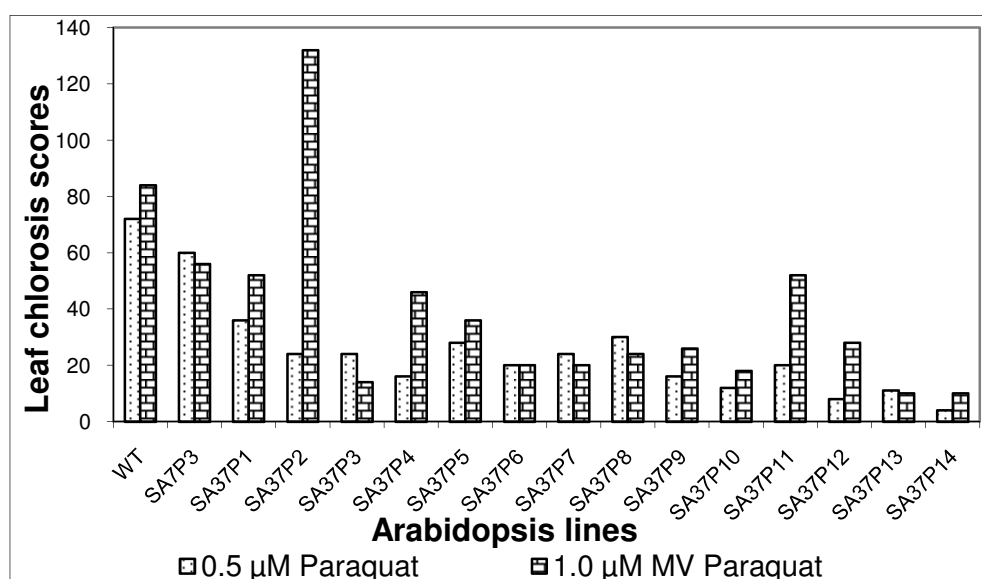


Fig. 3.2: Leaf scoring on the basis of loss of green colour under *in vitro* paraquat stress in ALDH3I1 and ALDH7B4 double mutants of *A. thaliana*.

On the basis of the results from these preliminary experiments it was decided to do further characterization of wild-type, single over-expressing line of ALDH3I1 (SA3P3), ALDH7B4 (SA7P3) and four dual over-expressing lines; SA37P6, SA37P10, SA37P13 and SA37P44.

3.1.2 RT-PCR

To test whether ALDH genes are expressed in leaves, a reverse transcription coupled to PCR (RT-PCR) analysis was used.

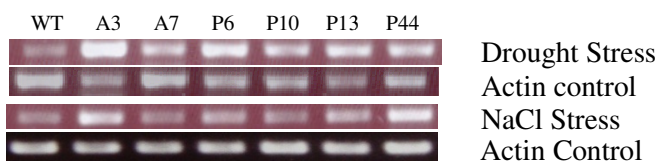


Fig.3.3. RT-PCR using ALDH3I1 gene specific primers. Here A3 and A7 are lines containing only ALDH3I1 and ALDH7B4; and P6, P10, P13 and P44 are double over-expressor lines

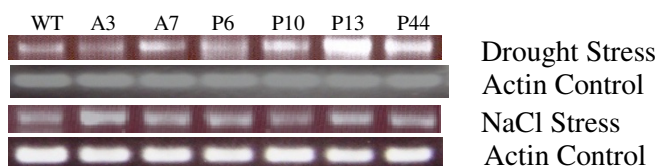


Fig.3.4. RT-PCR using ALDH7B4 gene specific primers. Here A3 and A7 are lines containing only ALDH3I1 and ALDH7B4 and P6, P10, P13 and P44 are double over-expressor lines

The RT-PCR also proved the more accumulation of mRNA of the two aldehyde dehydrogenase genes in the stressed plants as compared to the wild-type (untransformed) *Arabidopsis thaliana* plants (Fig. 3.3, 3.4).

RT-PCRs confirm the transcript expression of the two aldehyde dehydrogenase genes under stress conditions. The reverse transcription of RNAs into cDNAs and the amplification of the specific regions (500 bp) of ALDH3I1 and ALDH7B4 were done to detect the transcript expression even if it is in very small amount. Double over-expressing line (SA37P6) appeared to have the highest level of mRNA for ALDH3I1. All the transgenic lines, single as well as double over-expressing lines, had higher levels of transcript expression than wild-type *Arabidopsis*. ALDH7B4 expression was higher in the double over-expressing line SA37P13 whereas the other double over-expressing lines had lower expression levels than SA37P13 but still at a higher level than the single over-expressing line (SA7P3) and wild-type, under drought stress conditions.

Similarly, under salt stress (200 mM NaCl) the transgenic lines generally had higher ALDH mRNA levels than wild-type plants. ALDH3I1 mRNA expression was observed at the highest level in SA37P44 (double over-expressing line) whereas SA37P13 had almost the same level of expression as that of the single over-expressing line SA3P3 under high salt conditions. The double over-expressing lines SA37P6 and SA37P10 showed a bit less levels of mRNA expression than SA3P3 but they were still slightly higher than wild-type plants. Salt stress in double over-expressing lines did not seem to induce much higher levels of mRNA for ALDH7B4. Only a slight difference was observed among wild-type and transgenic lines under salt stress for ALDH7B4 transcript expression.

3.1.3 Protein Expression and Western Blots

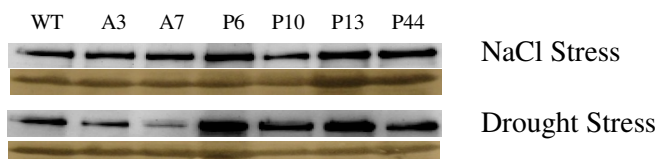


Fig. 3.5. Western blot (top) using ALDH3I1 antibodies from total proteins extracted; Ponceau-S staining (bottom) to check the equal loading of proteins. Here A3 and A7 are lines containing only ALDH3I1 and ALDH7B4; and P6, P10, P13 and P44 are double over-expressor lines

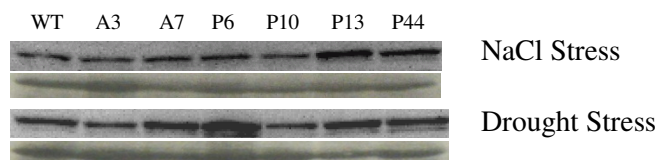


Fig.3. 6. Western blot (top) using ALDH7B4 antibodies from total proteins extracted; Ponceau-S staining (bottom) to check the equal loading of proteins. Here A3 and A7 are lines containing only ALDH3I1 and ALDH7B4; and P6, P10, P13 and P44 are double over-expressor lines

Western blots were done with wild-type as well as the transgenic lines revealed that the ALDH genes (ALDH3I1 and ALDH7B4) were expressed differentially. The antisera detected specific proteins bands of 60 kDa for ALDH3I1 and 54kDa for ALDH7B4. ALDH3I1 proteins expression was higher in the double over-expressing lines SA37P6, SA37P13 and SA37P44 while in one of the double over-expressing line (SA37P10) the expression of the proteins look similar to the single over- expressing line (SA3P3) under salt (200mM NaCl) stress. ALDH7B4 proteins are also expressed in salt-stressed plants but there the two double over-expressing lines (SA37P13 and SA37P44) had higher expression than wild-type and the other two double over-expressing lines (SA37P6 and SA37P10) had somewhat similar level of expression to the single over- expressing line (SA7P3)

The antibody for ALDH3I1, however, bound more specifically, under drought stress, indicating that the protein expression level is higher in all the double over-expressor plant lines. Here again the two lines, SA37P6 and SA37P13 expressed higher levels, resulting in more ALDH3I1 proteins as compared to the other two double over-expressing lines, single over-expressing line (SA3P3) and wild-type (Col0). In case of ALDH7B4 protein expression, the double over-expressing line SA37P6 showed the highest level of expression while the other three double over-expressing lines under study (SA37P6, SA37P10 and SA37P44) had expression level almost equal to the single over-expressing line (SA7P3) of ALDH 7B4 gene. Western blots revealed that in almost all of the salt (200

mM NaCl) as well drought stressed plants the ALDH3I1 and ALDH7B4 specific proteins were expressed.

3.1.4 Gas Exchange Measurements

In addition to molecular studies, some physiological studies were also done i.e., measurement of rate of gas exchange/photosynthesis. After one week of stress treatment CO₂ assimilation rate was measured (GFS- 3000; Heinz WALZ GmbH). The transformed plants showed higher assimilation rates (Fig. 3.7) than wild-type under both drought and salt stress.

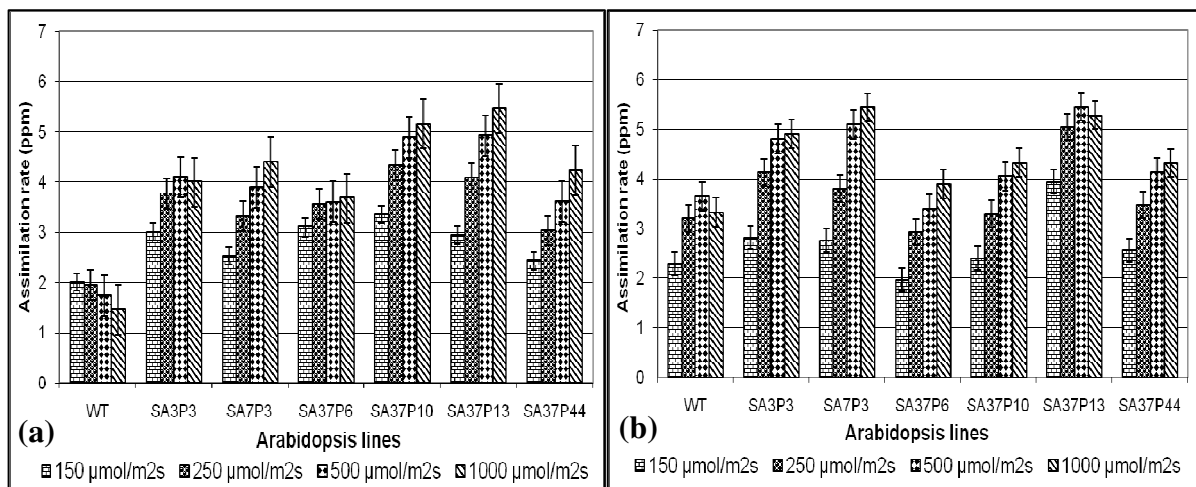


Fig. 3.7: (a) CO₂ assimilation rate after 1 week NaCl stress measured at various light intensities. (b) CO₂ assimilation rate after 1 week drought stress measured at various light intensities

The gas exchange measurements showed interesting results under salt stress conditions. The rate of assimilation ($\mu\text{mol}/\text{m}^2\text{s}$) of CO₂ observed at high illumination intensity (1000 $\mu\text{mol}/\text{m}^2\text{s}$) was lower in wild-type Arabidopsis plants (1.6 $\mu\text{mol}/\text{m}^2\text{s}$) while all the transgenic plants had much higher levels of assimilation rates, 4.0 $\mu\text{mol}/\text{m}^2\text{s}$ and 4.5 $\mu\text{mol}/\text{m}^2\text{s}$ in SA3P3 and SA7P3, respectively. The double over-expressors had even higher levels than the single over-expressing lines, i.e. 5.2, 5.5 and 4.2 $\mu\text{mol}/\text{m}^2\text{s}$ in SA37P10, SA37P13 and SA37P44, respectively. Only in line SA37P6 it was slightly lower (3.8 $\mu\text{mol}/\text{m}^2\text{s}$) than both single over-expressing lines. Interestingly, at lower light intensity (150 $\mu\text{mol}/\text{m}^2\text{s}$) differences among wild-type and transgenic plants were not so high (Fig. 3.7a). However, with increasing light intensity the difference in assimilation rates became more significant and transgenic plants proved to be performing better at higher light intensities under salt stress than wild-type.

Drought stress showed interesting impacts on the assimilation rates. At low light intensity (150 $\mu\text{mol}/\text{m}^2$) wild-type plants executed gas exchange of 2.3 $\mu\text{mol}/\text{m}^2\text{s}$ CO₂ which was

higher than the transgenic line SA37P6 (Fig. 3.7b). A similar trend was observed with salt-stressed plants; with increasing light intensities the wild-type plants had assimilated CO₂ less than transgenic plants and transgenic plants showed higher gas exchange levels at high light intensities. However, at the highest light intensity used (1000 $\mu\text{mol/m}^2\text{s}$) the highest assimilation rate (5.5 $\mu\text{mol/m}^2\text{s}$) was observed in single over-expressing line for ALDH7B4 (SA7P3) followed by SA37P13 (5.4 $\mu\text{mol/m}^2\text{s}$).

3.1.5 Lipid Degradation (MDA) Analysis

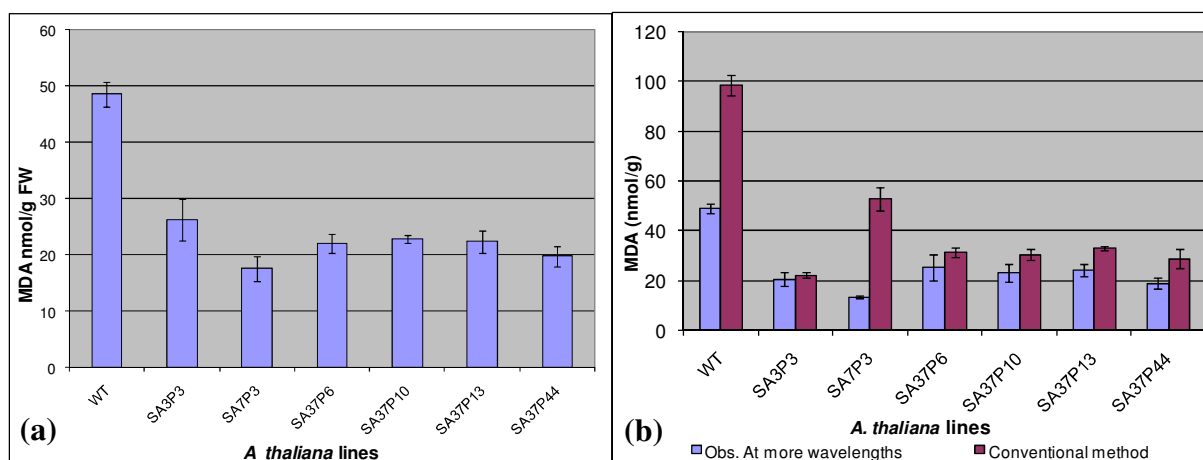


Fig. 3.8: (a): MDA measurement after a week of *in vitro* salt (150 mM NaCl) treatment in Arabidopsis ALDH lines. (b): Conventional vs. OD measurement at more wavelengths.

Malondialdehyde (MDA) is the end product of lipid per-oxidation. Therefore, to measure the levels of MDA in stressed plants is a very good bio-chemical indicator of stress tolerance in plants. MDA levels of plants grown on MS medium containing 150 mM NaCl were measured one week after stress. These measurements support the biochemical importance of aldehyde dehydrogenases. Wild-type Arabidopsis plants had much higher levels (49 nmol/g fresh weight) of MDA while single over-expressing lines of ALDH3I1 (SA3P3) and ALDH7B4 (SA7P3) had much lower MDA levels, 26.3 and 18.5 nmol/g fresh weight, respectively (Fig. 3.8a). The double over-expressing lines tend to have lower per-oxidation of lipids than ALDH3I1 single expressing line, indicating that they can withstand salt stress better than single over-expressing line of ALDH3I1 and much better than wild-type plants. Interestingly, all the double over-expressing lines under study had a little bit higher MDA levels than single over-expressing line (SA7P3), suggesting its superior performance.

There were some more interesting observations regarding MDA analysis, if conventional MDA measurement method used with measurements at only two wave lengths then wild-type showed even higher (about 100 nmol/g) MDA levels. While SA7P3 also showed higher MDA levels than other transgenic lines (Fig. 3.8b). Another interesting observation was the higher levels of MDA in transgenic plants or sometimes no difference in MDA levels, if the period of stress was prolonged i.e. longer than 12 days or more.

3.1.6 Chlorophyll Content Measurements

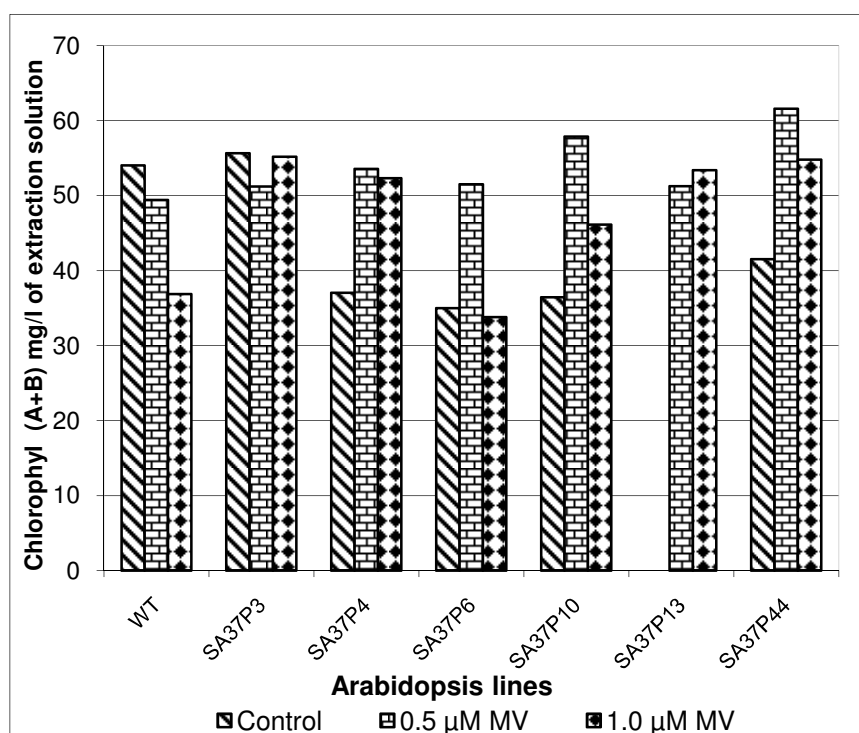


Fig.3.9: Chlorophyll content of leaves as effected by different levels of Methyl Viologen (MV)

Chlorophylls are important pigments of the photosynthetic apparatus. The higher the chlorophyll content the higher is photosynthesis due to the more amount of light energy captured. The total chlorophyll content of the plants under stress was measured after 24 hrs of dipping (submerging) plants at various levels (0, 0.5, 1.0 μ M) of methyl viologen (MV) or paraquat. At 0.5 μ M of MV there was only a slight difference of total chlorophyll content among wild-type and transgenic plants (Fig. 3.9). However, the double over-expressing lines SA37P10 and SA37P44 had higher levels of chlorophyll. At 1.0 μ M of MV transgenic lines proved to retain the chlorophyll content more than wild-type but one double expressing line SA37P6 had almost the same amount of chlorophyll than wild-type plants.

3.1.7 Prolonged Drought Stress

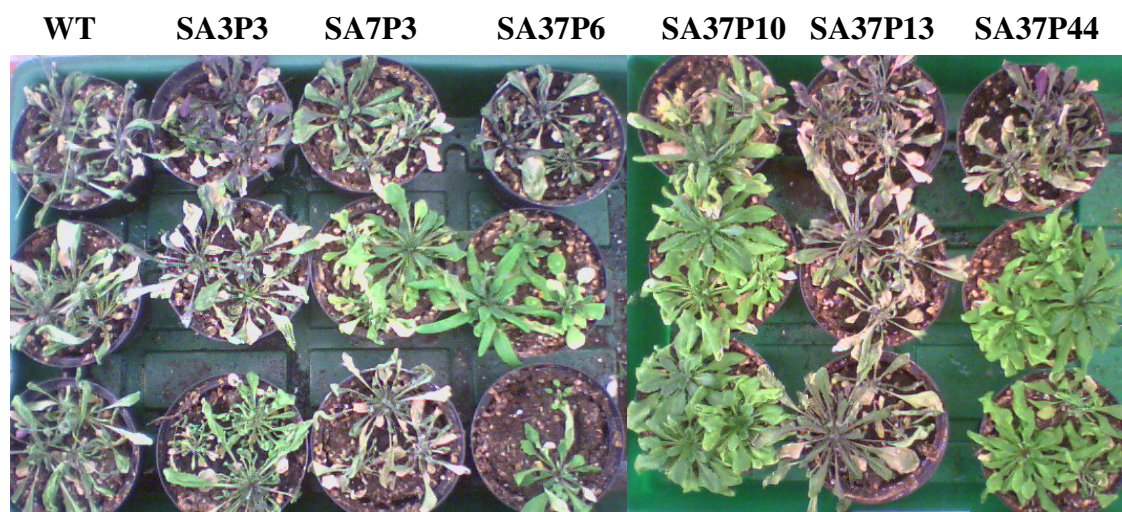


Fig. 3.10: Phenotype of wild-type and transgenic plants of *A. thaliana* after 3 weeks of drought stress. n=10/line

The threshold of plants to withstand the prolonged drought was tested by withholding water for more than 3 weeks. Most of the lines including wild-type were completely wilted but almost all the plants of the double over-expressing line SA37P10 survived and 2/3 of the plants of the line SA37P44 also seemed to tolerate longer periods of drought (Fig. 3.10). The experiments showed a clear ability of the tolerance of double over-expressing lines to prolonged drought spells.

3.1.8 Tobacco ALDH lines

To investigate the potential of the ALDH genes further, ALDH3I1 ALDH7B4 genes were transformed into tobacco (*Nicotiana tabacum*). The plants resulting from the callus were verified for the presence of transgenes (ALDH3I1 and ALDH7B4) via genomic PCRs (Fig.3.11) and PCR positive plants were considered to be transgenic.

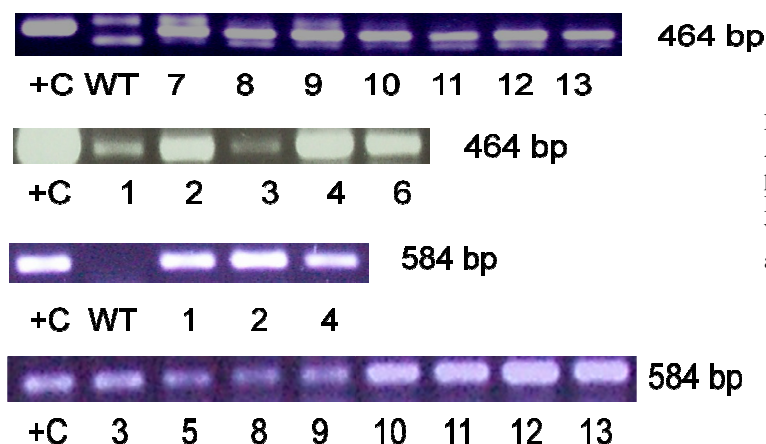


Fig. 3.11: PCR of ALDH 3I1 (464 bp) and ALDH7B4 (584bp) genes from tobacco plants. +C = (positive control, Plasmid DNA of the vector carrying the gene), WT= Wild-type and rest are the numbers assigned to the transgenic lines

3.1.9 Transcript Expression in Tobacco ALDH Lines

To check the expression of ALDH3I1 and ALDH7B4 transcripts, total RNA was extracted from wild-type plants as well as transgenic lines growing under control conditions. Northern blots were done according to the section (2.24). The result of the northern blot shows (Fig. 3.12 and 3.13) the presence of the transcripts for ALDH3I1. The transcript was not detected in wild-type (SR1) plants. Among the transgenic plants some lines (SA3P4, SA3P7, SA3P10, SA3P12 and SA3P14) the transgene was expressed at a high level. On the contrary, lines SA3P3 and SA3P7 did not show any expression at all, however, genomic PCR had confirmed the presence of the gene (ALDH 3I1) in the plants. The other lines showed medium level of expression.

The transcript expression of ALDH7B4 behaved strange. Despite the fact that gene specific primers did not amplify a correct band in wild-type, at transcript level it was not true. Apparently all the lines including wild-type plants showed a medium level of transcript that seemed to be of the same level, indicating that there is some endogenous (or homologue to ALDH7B4) ALDH7B4 in tobacco

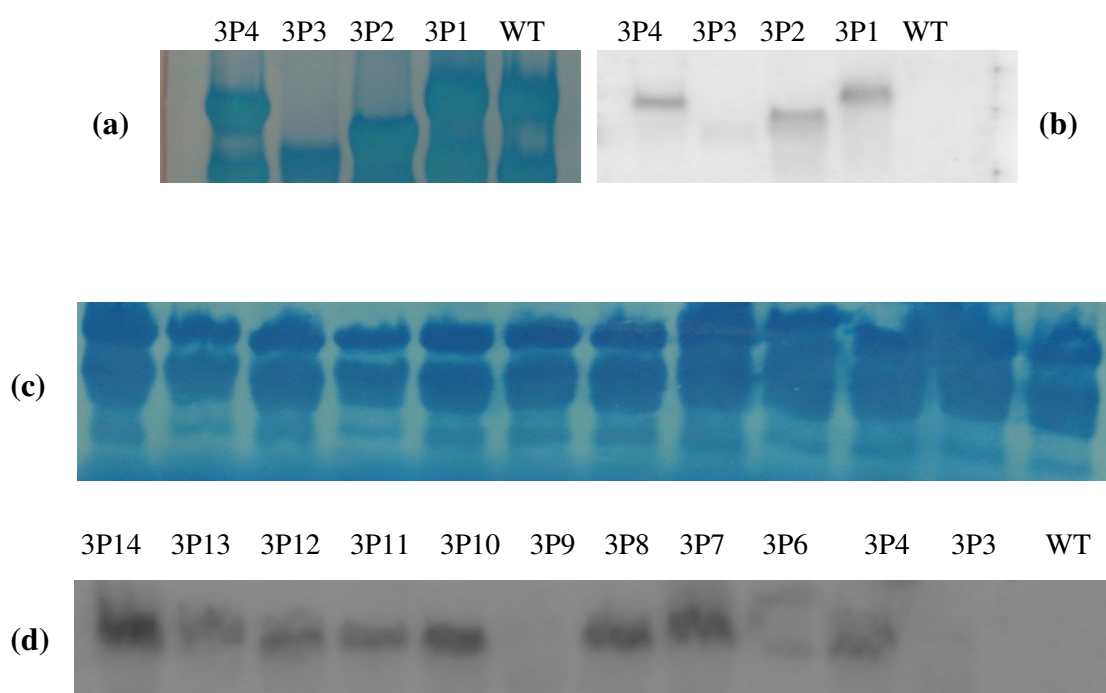


Fig. 3.12: (a,c) Nylon membrane stained with methyl blue after blotting and before probe hybridization. (b,d) Northern blots hybridized with radio labelled ALDH3I1 probe.

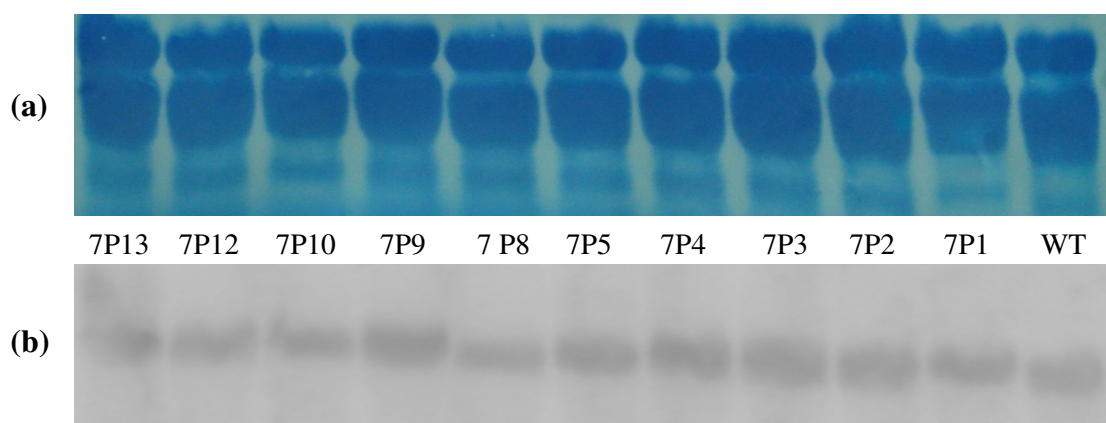


Fig. 3.13: (a) Nylon membrane stained with methyl blue after blotting and before probe hybridization. (b) Northern blots hybridized with radio labelled ALDH7B4 probe.

3.1.10 Plant Growth under *In Vitro* Salt Stress

To select better performing transgenic lines, *in vitro* salt treatments (200 mM and 400 mM NaCl) were tried. At 400 mM NaCl all plants of all lines died after 2 weeks. At 200 mM NaCl the different lines responded differentially. Many transgenic lines grew better on MS+200 mM NaCl medium than wild-type (Fig. 3.14). Only 57 % wild-type plants could grow on MS with salt medium. Moreover, they also had about 27 % yellow leaves. Plants of transgenic lines (SA3P12 and SA7P9) exhibited growth up to 83 % the best salt tolerance ability was observed in ALDH7B4 line SA7P13 for which 85% plants showed growth on the salt medium. However, the same transgenic lines were inferior to wild-type (untransformed) plants showing only 52 % (SA3P8) growth under salt stress.

Fig. 3.14 also shows the mean number of leaves turned yellow after the salt stress. Here again some transgenic lines proved to be superior to wild-type plants. The plants grown from wild-type had 27 % yellow leaves while some transgenic lines; SA3P12 had only 11.17%, SA7P13 with 15.17% had very low percent of leaves turned yellow. here were also a few transgenic lines which could not tolerate stress better than wild-type plants and even had up to 58 % (SA3P8) yellow leaves.

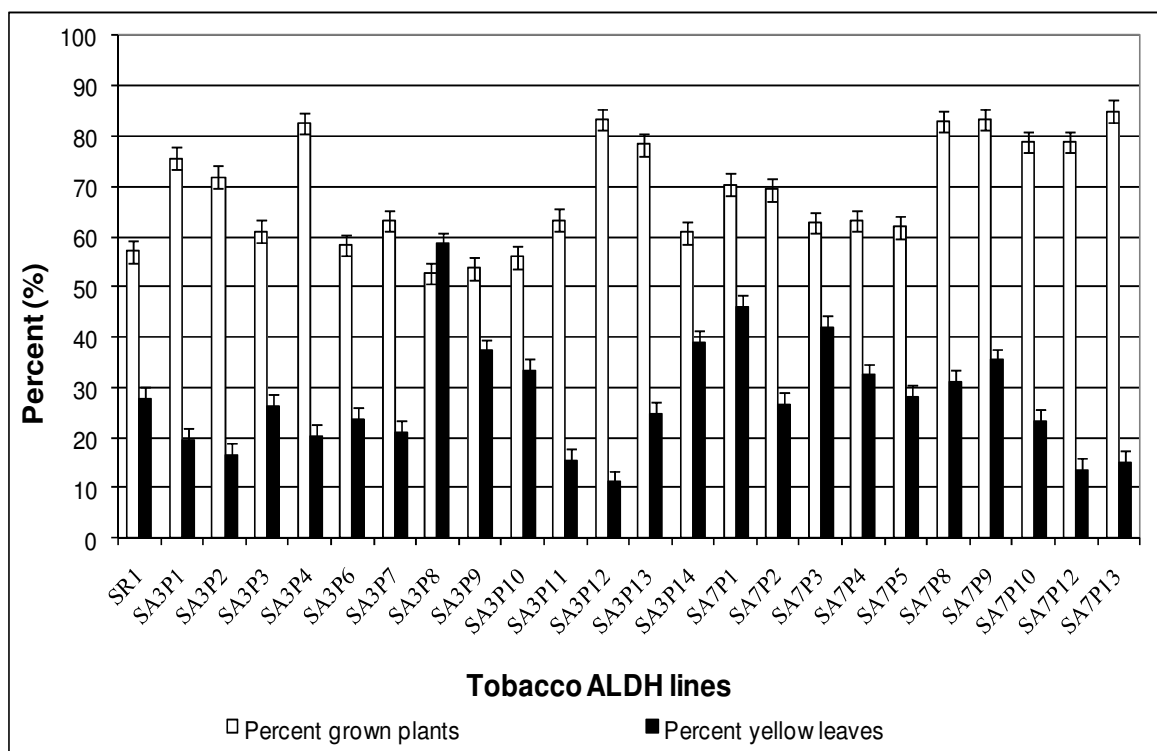


Fig. 3.14: Tobacco plant growth under *in vitro* salt stress expressed as percent of plants grown and percent of yellow leaves on them. Data represent means values \pm SE.

3.1.11 *In Vivo* Detection of H₂O₂ under Oxidative Stress

Leaf discs (12mm) of tobacco plants were incubated in different levels of methyl viologen (0, 2, 5 and 10 μ M) for 24 hours to study the tolerance of plants towards oxidative stress (Fig. 3.15). A very good indicator of oxidative stress is the analysis of reactive oxygen species (ROS) such as H₂O₂ and O⁻². H₂O₂ was detected using the DAB uptake method. Wild-type plants tolerated lower levels (2 μ M) of methyl viologen or paraquat equal to some transgenic plants (Fig. 3.13). However, with increasing levels of paraquat, leaf discs from wild-type plants did not show stress tolerance that resulted as production of more amount of H₂O₂ (dark brown spots/areas indicated the site of H₂O₂ production). In addition to wild-type some transgenic lines (SA3P4, SA3P6, SA3P10, SA3P12 and SA3P14) also did not show complete tolerance to higher (10 μ M) level of paraquat. In general, all transgenic lines tested tolerated a medium level (5 μ M) of paraquat.

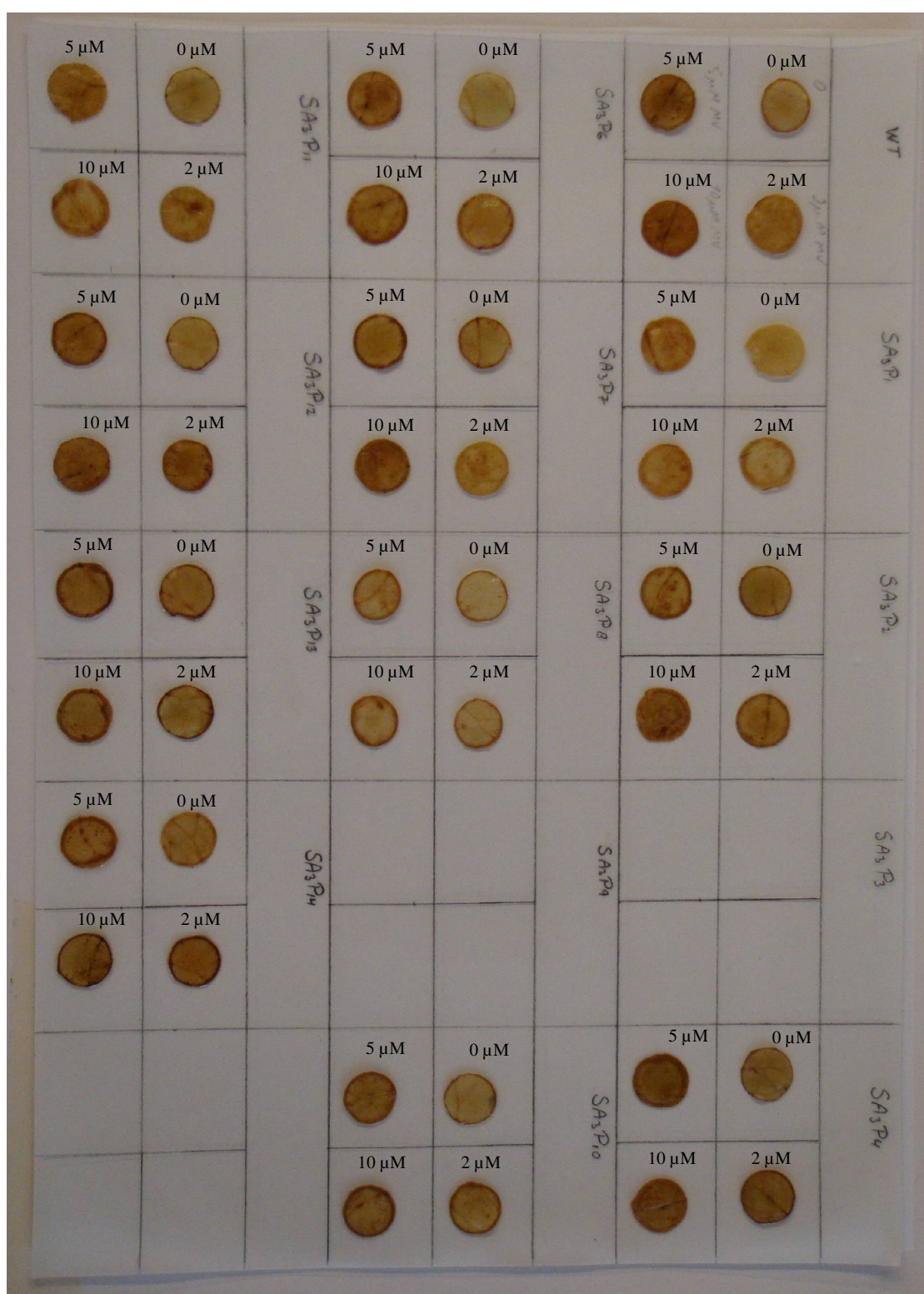


Fig. .3.15: Leaf discs showing localized detection of H_2O_2 as brown spots (areas) after treatment of various levels of methyl viologen. For each line the leaf-dics were dipped in 0 μ M, 2 μ M, 5 μ M and 10mM paraquat.

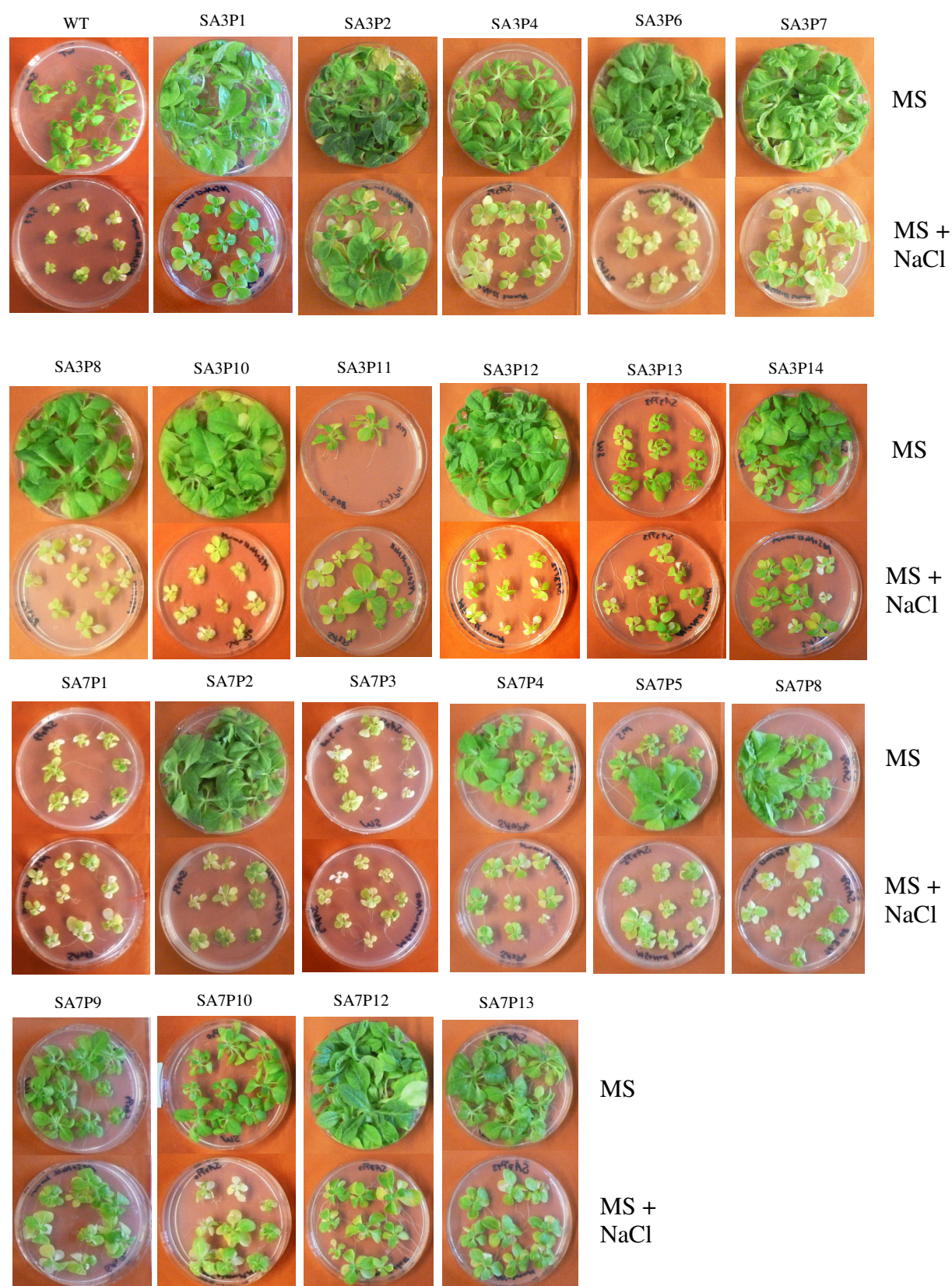


Fig. 3.16: Phenotype of the tobacco ALDH lines after 4 weeks grown on MS (top pic. of each line) vs MS+200 mM NaCl medium (lower pic. of each line).

3.1.12 Root Length under *In Vitro* Salt Stress

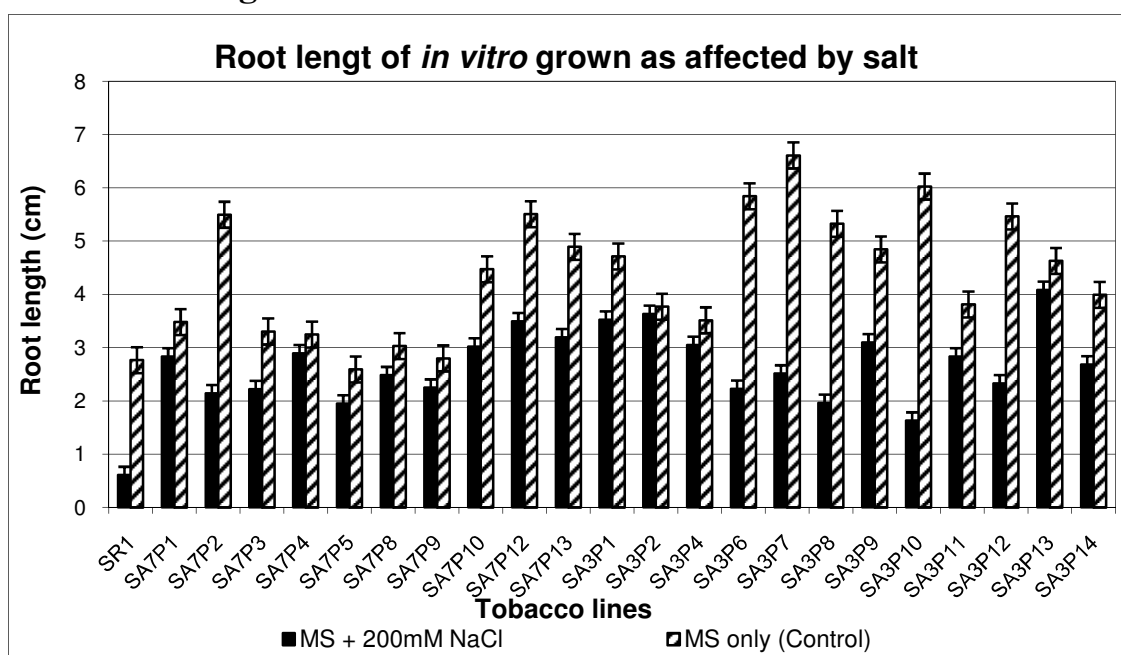


Fig. 3.17: Root length of 5-6 week old *in vitro* grown tobacco wild-type and transgenic plants grown in the presence of 200 mM NaCl for 3-4 weeks compared with plants grown on control (MS) medium. Data represent the mean values \pm SE.

In vitro grown plants presented a very good means of screening for tolerance to high salt levels (200 mM and 400 mM NaCl). However, at 400 mM all plants of wild-type as well as all transgenic lines turned completely yellow. Therefore, further screening was done only on control media (MS) and MS + 200 mM NaCl. Even under normal conditions (MS medium only) the roots of wild-type plants did grow less (2.7 cm). Most of the transgenic lines had longer roots than wild-type. Only lines SA7P5 (2.6 cm) had shorter roots than wild-type (SR1) plants. Some lines (SA3P7, SA3P10) had more than twice the length of roots than wild-type, on MS medium only (Fig. 3.17). High and prolonged salt stress seemed to affect the root growth of wild-type plants drastically and the plants did not continue to grow normally, resulting in $\frac{1}{4}$ decreases (0.7 cm) in root length. On the other hand, in transgenic lines generally the root length was not as badly affected as in wild-type. For some lines, however, root length was not much affected e.g. SA3P13 had root length of 4.6 cm under normal conditions while under salt medium it was 4.08 cm. Similarly, SA3P2 showed also less decrease in root length under salt medium as compared to MS medium. In some lines the root length was reduced under salinity conditions but their root lengths were even more than those of wild-type plants.

3.1.13 Seedling Length under *In Vitro* Salt Stress

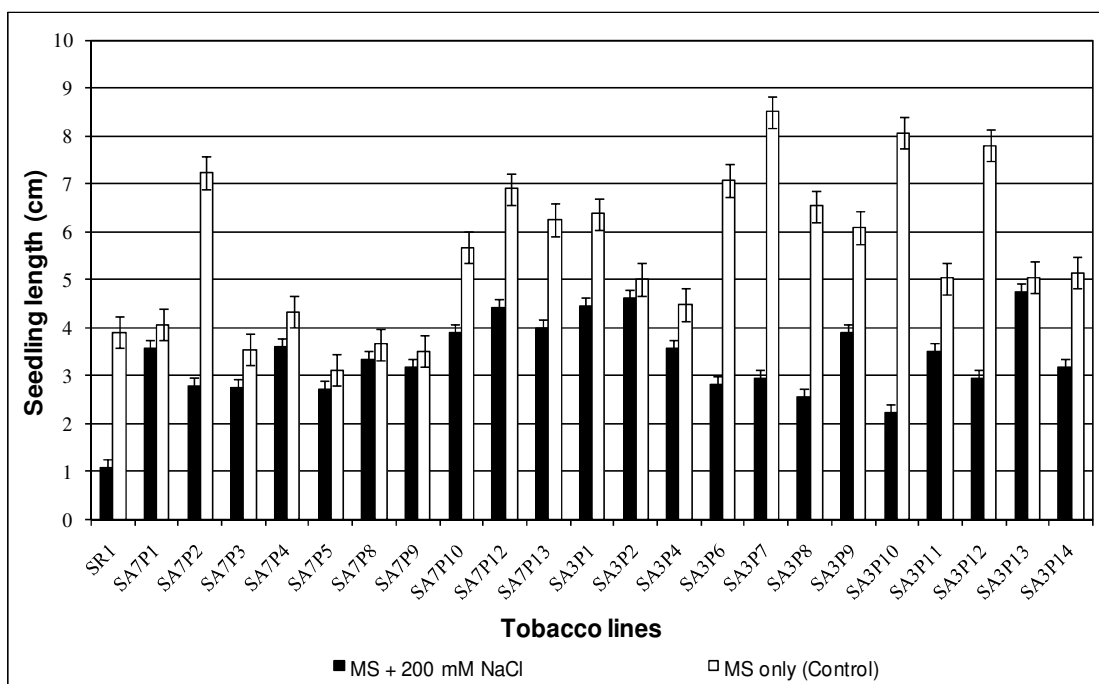


Fig.3.18: Seedling lengths of 5-6 week old *in vitro* grown tobacco wild-type and transgenic plants grown in the presence of 200 mM NaCl for 3-4 weeks compared with plants grown on control (MS) medium. Data represent the mean values \pm SE.

Similar changes as for roots were observed for total seedling lengths under control and high salt (MS+200mM NaCl) medium. Where seedling/plant length was 4 fold decreased (3.9 cm to 1.1 cm) in wild-type plants. Whereas, all transgenic lines had longer seedling length (height) as compared to wild-type plants (Fig. 3.18). Here again SA3P13 was least effected by salt stress and the length in seedling was not much changed on MS + 200 mM NaCl medium. The lines SA3P7, SA3P10, SA3P12 and SA7P2 exhibited much decrease in root seedling length under salt conditions. These phenotypic changes were observed twice and made it easier to select lines that expressed more levels of the transgenic ALDH3I1 or ALDH7B4.

3.1.14 Ion Leakage under Abiotic Stresses

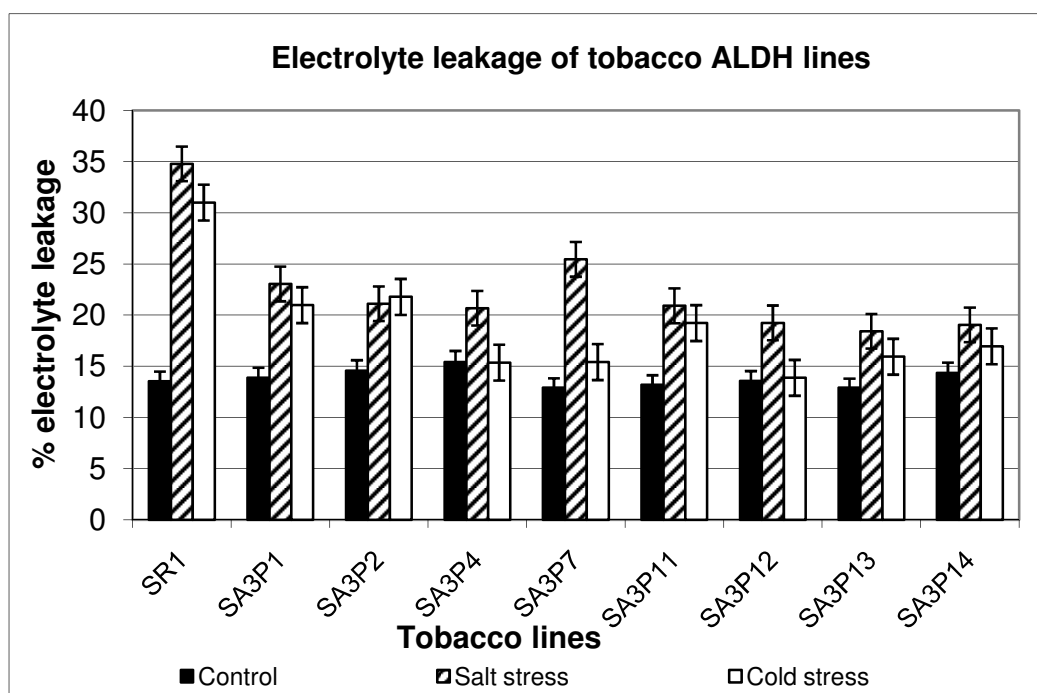


Fig. 3.19: Electrolyte leakage of tobacco ALDH311 lines under control, 2 weeks cold stress and 2 weeks salt (200 mM NaCl) expressed as percent, measurements were taken before and after boiling the leaf disc solutions. Data represent the mean values \pm SE.

Membrane damage can be measured by measurement of electric conductivity of the leaf solution and is expressed as the difference of electric conductivity before (actual) and after boiling (total). The electrolyte leakage percentage was almost the same for wild-type and transgenic tobacco plants under control growth conditions. However, under stress conditions the electrolyte leakage was higher in wild-type plants as compared to transgenic plants. Two weeks cold stressed, wild-type tobacco plants had 30% electrolyte leakage (Fig. 3.19). Whereas, the electrolyte leakage values among transgenic lines was maximum 21% (line SA3P2). In lines SA3P4 and SA3P12 electrolyte leakage remained also almost unchanged, under cold stress.

Salt stress had a little bit more adverse effects on electrolyte leakage in wild-type and it was measured to be up to 35%. The highest electrolyte leakage among transgenic lines was 25% in line SA3P7. Transgenic lines SA3P11, SA3P12 and SA3P14 showed less electrolyte leakage than the other transgenic lines.

3.1.15 Lipid Peroxidation (MDA) Analysis under Oxidative Stress

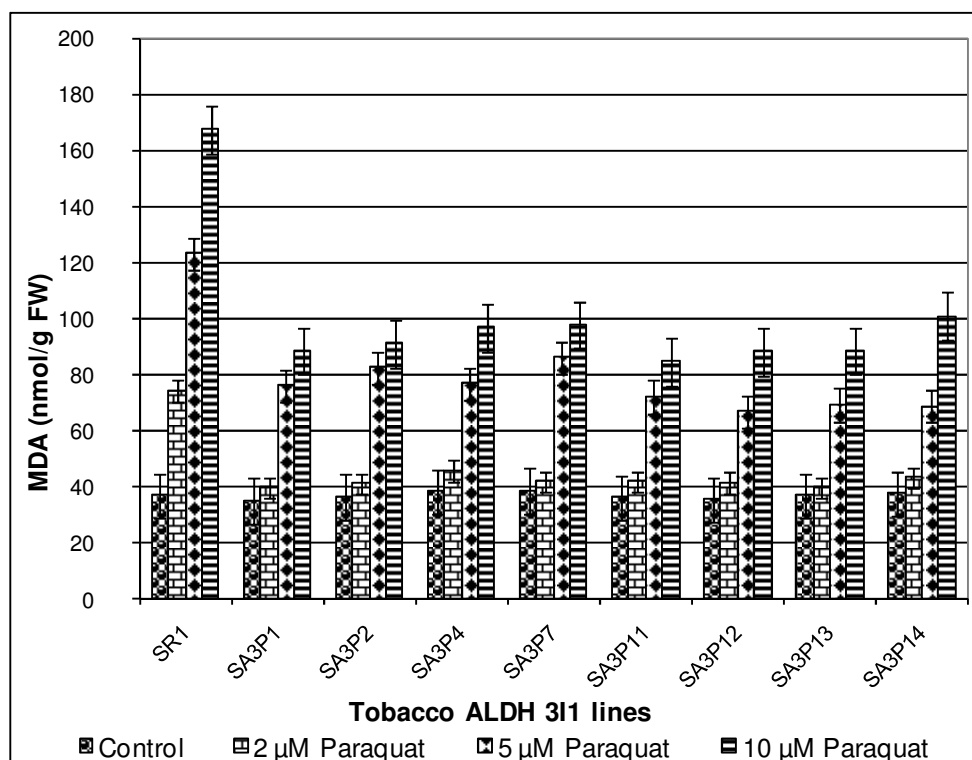


Fig. 3.20: Lipid per-oxidation measurement as MDA (nmol/g FW) of the leaf discs under oxidative stress; several levels of paraquat were tested. Data represent the mean values \pm SE.

Under oxidative stress, induced by different levels of methyl viologen or paraquat, the lipid per oxidation was also determined. In control treatment (0 μ M paraquat) leaf discs were in only water. The amount of malondialdehyde (MDA) was not much different for all the lines, when leaf discs were in water (Fig 3.20). The presence of even lowest level of paraquat (2 μ M) drastically increased lipid-per-oxidation. It was almost double (69.1 nmol/g) in wild-type. The levels of MDA also increased in transgenic plants; however, at 2 μ M paraquat the increase was not as much as compared to the wild-type. Higher levels of paraquat (5 μ M) further increased (almost four times the level under control) the MDA levels to 120.9 nmol/g. At this level of paraquat transgenic plants also had higher levels of MDA (85.8 nmol/g in SA3P7) but it was still lower than wild-type. 10 μ M paraquat treatment resulted in a very high level of MDA in wild-type (167.2 nmol/g) going up to almost 6 times the level under control treatment. However, the increase in MDA levels in transgenic plants was not as much as in wild-type. Maximum MDA levels were observed in the transgenic line SA3P14 and SA3P7 i.e. 100.6 nmol/g and 97.6 nmol/g, respectively. These levels were almost two third of the MDA level in wild-type plants.

3.1.16 Chlorophyll Content (a+b) Measurements under Oxidative Stress

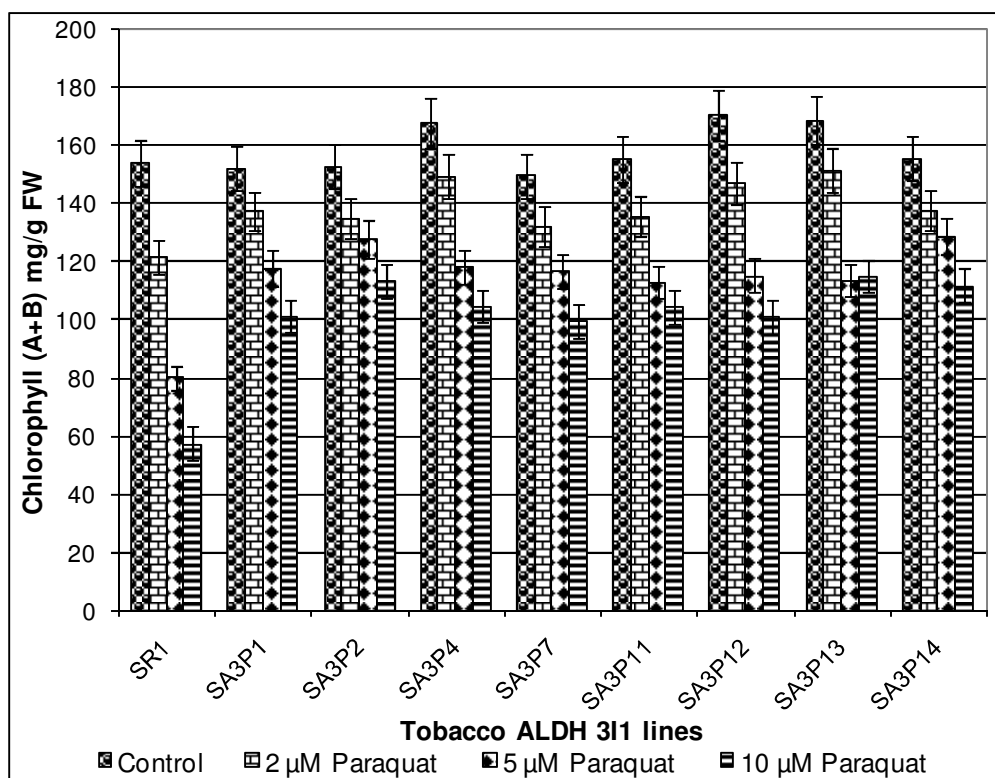


Fig. 3.21: Chlorophyll (a+b) content measurements as mg/g fresh weight of the leaf discs under oxidative stress; several levels of paraquat were tested. Data represent the mean values \pm SE.

Oxidative stress damages many cell organelles and to measure the amount of the photosynthetic pigment (chlorophyll) also gives an idea of the ability of transgenic plants to cope with stress conditions. Tobacco leaf discs under control conditions had a higher level of total chlorophyll (A+B) ranging 152.35 to 170.14 mg/g of fresh weight (FW). In the presence of paraquat the amount of chlorophyll decreased. The magnitude of decrease was higher in wild-type leaf discs than in the leaf discs from transgenic plants. At only 2 μ M paraquat the amount of chlorophyll in wild-type was reduced to 121.6 mg/g while all transgenic plants showed only a slight decrease in chlorophyll content (Fig. 3.21). Under 5 μ M paraquat treatment there was a drastic decrease in chlorophyll levels in wild-type to almost half (80.1) of the control level. However, the transgenic plants performed much better than wild-type showing only a slight decrease in chlorophyll levels ranging from 113.7 to 130.3 mg/g of fresh weight. The highest level of paraquat tested (10 μ M) proved to be the most devastating for the chlorophyll level in wild-type and it was decreased down to 57.4 mg/g (almost one third of control). In transgenic lines, the chlorophyll did not decrease as much as in the wild-type. The lowest chlorophyll (97.7 mg/g) level among

transgenic lines was observed in line SA3P12, which was almost two fold higher than wild-type.

3.1.17 Gas Exchange Measurements

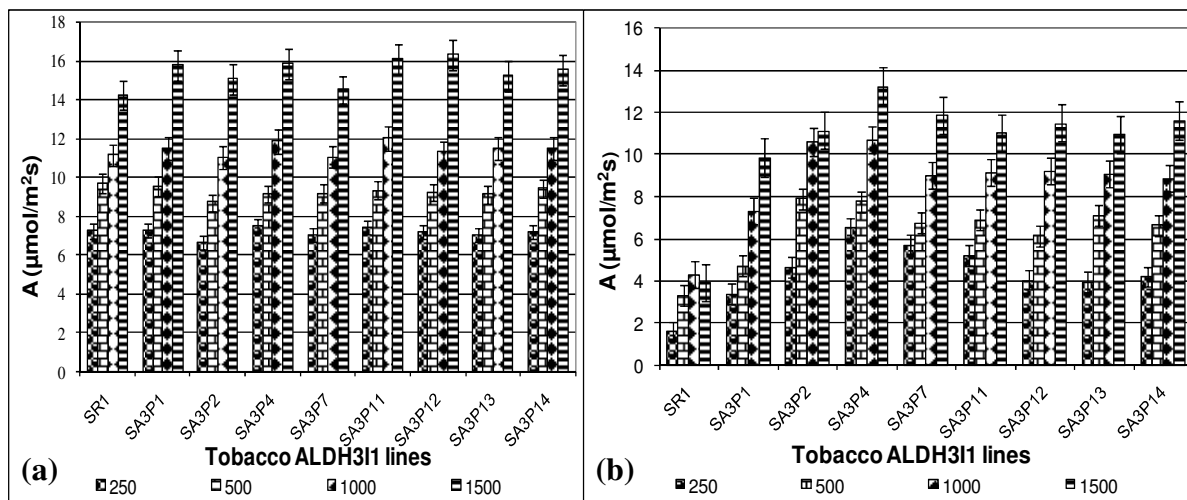


Fig. 3.22: (a) CO₂ assimilation rate under control/normal growth conditions in tobacco lines measured at various light intensities. (b) CO₂ assimilation rate after 2 weeks drought stress measured at various light intensities

Gas exchange measurements were also done with tobacco plants under normal conditions and abiotic stress conditions; salt, drought and cold. Gas exchange under normal conditions at low light intensity (250 $\mu\text{mol}/\text{m}^2\text{s}$) was observed in a range of 6.5 to 7.2 $\mu\text{mol}/\text{m}^2\text{s}$ (non-significantly different) in wild-type and transgenic lines (Fig. 3.22a). However, drought stress reduced the gas exchange in wild-type to less than half of that under normal conditions at low light intensity (250 $\mu\text{mol}/\text{m}^2\text{s}$). The transgenic lines also showed reduced gas exchange but that decrease was not as adverse as in wild-type plants (Fig. 3.22b). Among transgenic lines SA3P4 executed the highest (6.2 $\mu\text{mol}/\text{m}^2\text{s}$) gas exchange at 250 $\mu\text{mol}/\text{m}^2\text{s}$ that was not much decreased as compared to gas exchange under normal conditions in that line. At light intensity of 1000 $\mu\text{mol}/\text{m}^2\text{s}$ the assimilation rate was not significantly different among wild-type and transgenic lines under normal conditions. At this light intensity drought stress had a great impact on photosynthetic efficiency of wild-type and CO₂ assimilation as it was reduced to almost 1/3. Transgenic lines, however, showed a better performance; lines SA3P2 and SA3P4 executed much less changes in the CO₂ assimilation rate under drought conditions at 1000 $\mu\text{mol}/\text{m}^2\text{s}$ light intensity. Similarly, under light intensity of 1500 $\mu\text{mol}/\text{m}^2\text{s}$ there was less decrease in the CO₂ assimilation rate in transgenic plants as compared to wild-type plants.

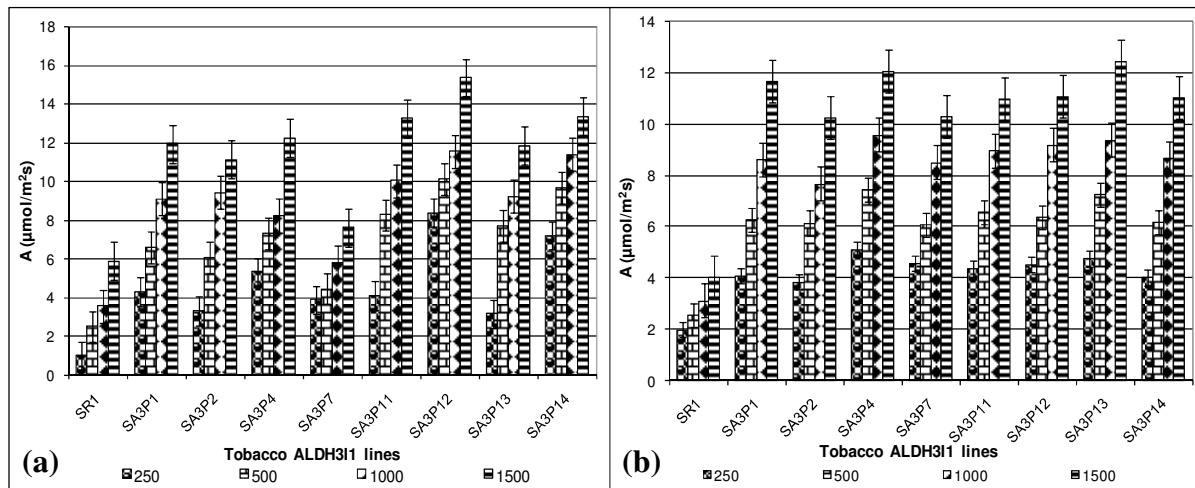


Fig. 3.23: (a) CO₂ assimilation rate after 2 weeks of cold stress in tobacco lines measured at various light intensities. (b) CO₂ assimilation rate after 2 weeks salt (200 mM NaCl) stress measured at various light intensities

Photosynthesis measurement under cold stress revealed similar results. The gas exchange in wild-type plants was more affected than under drought stress. At low light intensity (250 $\mu\text{mol}/\text{m}^2\text{s}$) gas exchanged was reduced to almost 6 folds (Fig. 3.23a) in wild-type. Transgenic lines on the other hand, showed not such a drastic decrease in CO₂ assimilation. Lines SA3P12 and SA3P14 had almost unaltered gas exchange rate under cold stress. Wild-type plants could only assimilate almost 5-fold less CO₂ under cold stress at light intensity of 500 $\mu\text{mol}/\text{m}^2\text{s}$. Transgenic lines had higher assimilation as compared to wild-type and in the lines SA3P11, SA3P12 and SA3P14 there was almost no change in CO₂ assimilation under cold stress. At light intensity of 1000 $\mu\text{mol}/\text{m}^2\text{s}$ wild-type could assimilate 1/3 CO₂ as compared to control conditions, whereas the transgenic lines had much higher assimilations rates than wild-type plants. In the lines SA3P12 and SA3P14 there was almost no change in gas exchange under cold stress and control conditions. Wild-type plants showed half of the CO₂ assimilation rate at light intensity of 1500 $\mu\text{mol}/\text{m}^2\text{s}$ as compared to normal conditions. On the other hand, transgenic lines did not suffer such a big change in CO₂ assimilation rate. In the line SA3P12 there was only a slight downward shift in gas exchange.

Assimilation in wild-type plants under salt stress sharply decreased, minimizing to 2.0 $\mu\text{mol}/\text{m}^2\text{s}$ only, at 250 $\mu\text{mol}/\text{m}^2\text{s}$. The transgenic lines had not shown such a drastic decrease in CO₂ assimilation (Fig. 3.23b). At light intensity of 500 $\mu\text{mol}/\text{m}^2\text{s}$ transgenic lines showed some decrease in assimilation rates. The lines SA3P4 and SA3P13 had higher assimilation rates (7.3 and 7.2 $\mu\text{mol}/\text{m}^2\text{s}$, respectively) than other transgenic lines and

wild-type had more than a 4 fold decrease in assimilation rate. Assimilation in wild-type plants at 1000 $\mu\text{mol}/\text{m}^2\text{s}$ was minimized to a quarter as compared to control and half to one third as compared to the transgenic lines. Among transgenic lines SA3P12 and SA3P13 were the best performing lines at 1000 $\mu\text{mol}/\text{m}^2\text{s}$. Wild-type plants, however, had lower CO_2 assimilation rates at 1500 $\mu\text{mol}/\text{m}^2\text{s}$ under salt stress as compared to cold stress (4 $\mu\text{mol}/\text{m}^2\text{s}$) and much lower to that under control conditions. On the other hand all transgenic lines had a very high assimilation rate. Among them SA3P13 and SA3P4 had higher assimilation rates (12.6 and 12.1 $\mu\text{mol}/\text{m}^2\text{s}$) which were not much different from assimilation under control conditions.

3.1.18 Chlorophyll Fluorescence Measurement

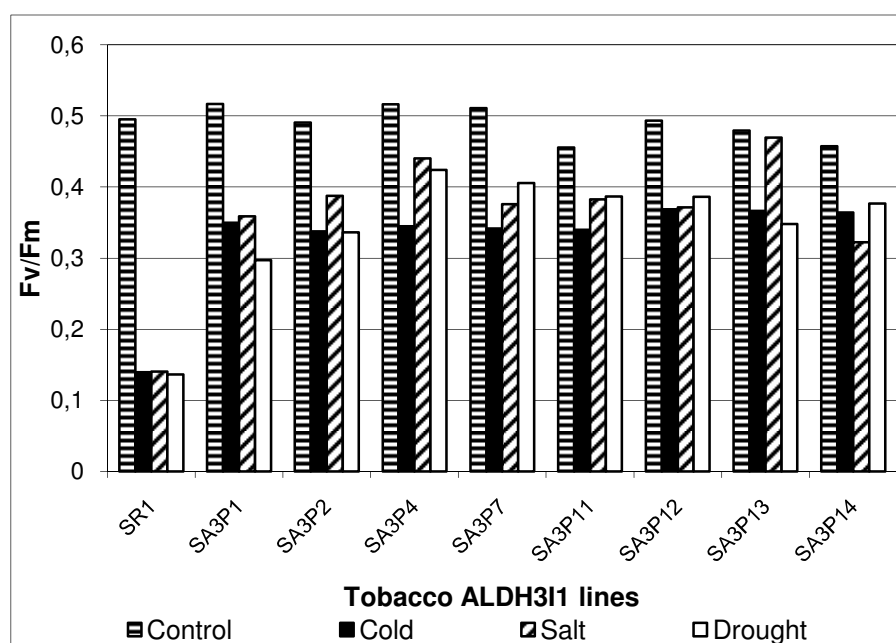


Fig. 3.24: Comparison of chlorophyll fluorescence of tobacco ALDH 3I1 lines under control and various abiotic stress condition; 2 weeks cold, 2 weeks salt and 2 weeks drought stress.

The chlorophyll fluorescence of wild-type and transgenic plants showed almost similar values under control conditions. However, under cold, salt and drought stress conditions the F_v/F_m ratio was reduced to almost a quarter as compared to control. Under cold stress SA3P12 and SA3P13 were the transgenic lines with the highest chlorophyll fluorescence, 0.38 and 0.39, respectively. Under salt stress conditions, SA3P13 performed very well and had a chlorophyll fluorescence ratio of 0.48 (Fig. 3.24). The line SA3P4 lagged behind it with a chlorophyll fluorescence value of 0.44. Similarly, under drought stress transgenic plants had much higher values of chlorophyll fluorescence compared to wild-type plants.

Line SA3P4 was the line with the highest chlorophyll fluorescence value of 0.42 under drought stress.

3.1.19 Chlorophyll Content (a+b) under Abiotic Stresses

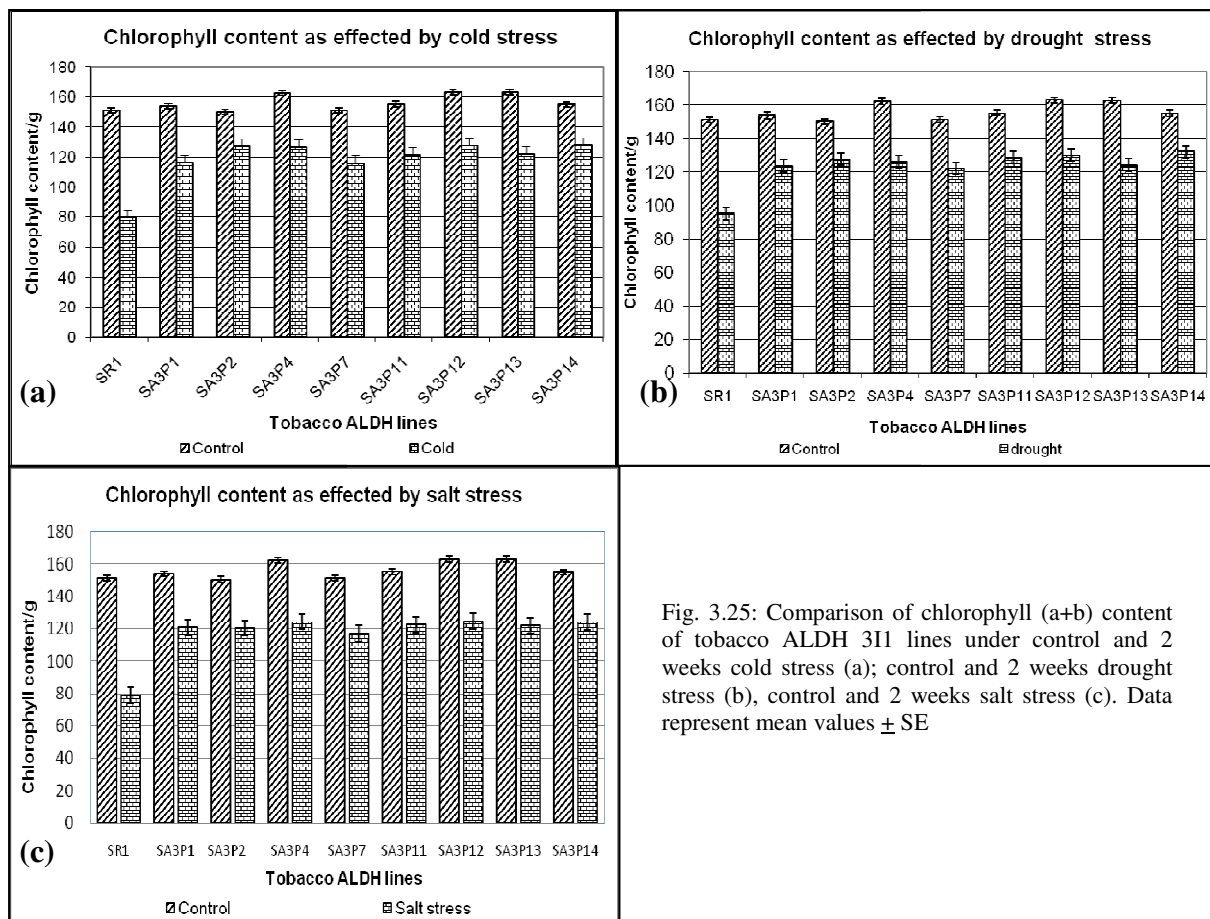


Fig. 3.25: Comparison of chlorophyll (a+b) content of tobacco ALDH 311 lines under control and 2 weeks cold stress (a); control and 2 weeks drought stress (b), control and 2 weeks salt stress (c). Data represent mean values \pm SE

The chlorophyll (a+b) content of the wild-type and transgenic plants was measured under control conditions as well as under cold, drought and salt stress. Under control conditions there were non-significant differences in chlorophyll content of wild-type and transgenic lines. However, under cold conditions the chlorophyll content of the wild-type plants decreased to 2/3 of that of control conditions. The transgenic lines also showed a reduced value of chlorophyll content but the decrease was not as sharp as in wild-type plants (Fig. 3.25). Transgenic lines had 120 to 124 mg chlorophyll/g of fresh weight of leaves after drought stress the chlorophyll content of wild-type was 97 mg chlorophyll/g fresh weight of plant. On the other hand the transgenic lines had chlorophyll contents varying from 120 (SA3P7) to 126 mg/g (SA3P2). Salt stress had reduced the chlorophyll content of wild-type plants to almost half (80 mg/g fresh weight). The transgenic plants again had higher chlorophyll contents ranging from 119 mg/g FW (SA3P7) to 122 g/g FW (SAP14).

3.1.20 Lipid Peroxidation (MDA) Analysis under Abiotic Stresses

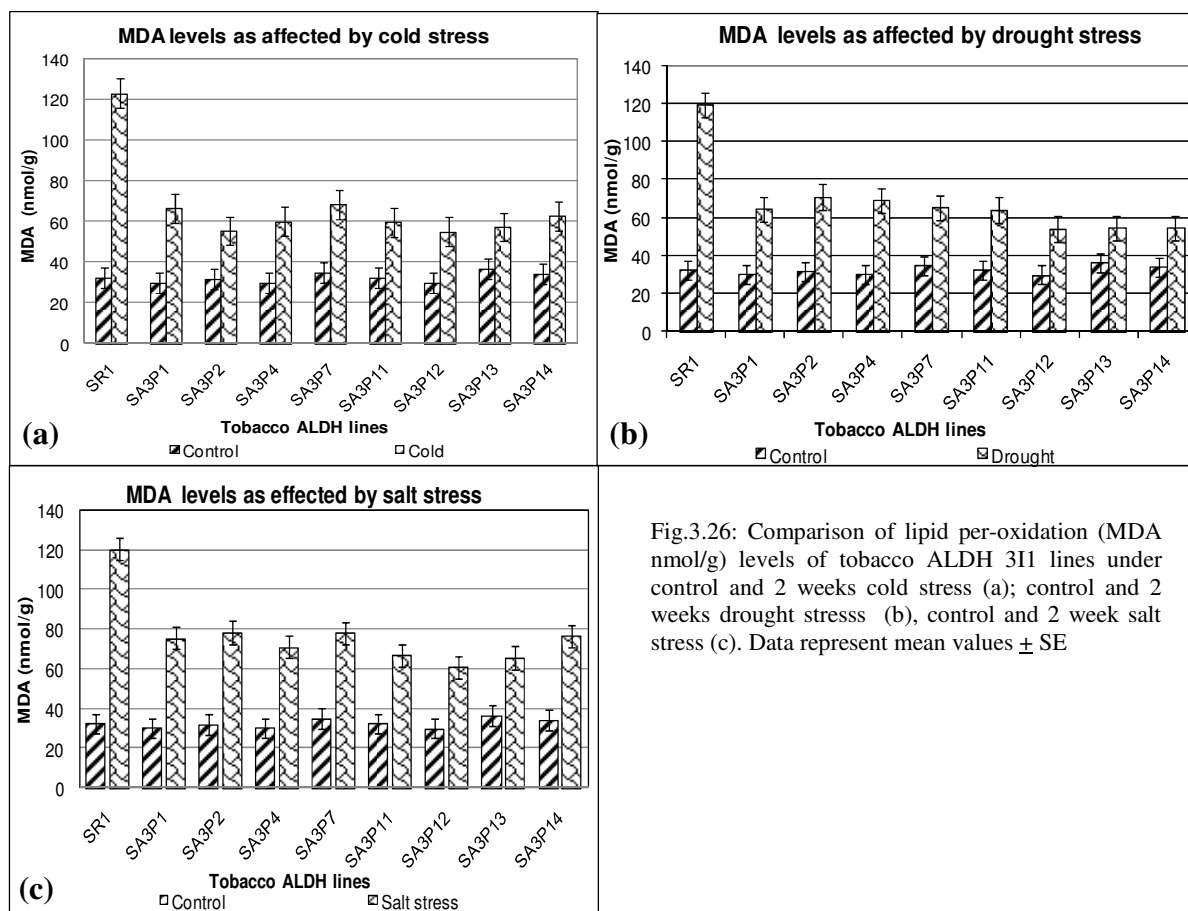


Fig.3.26: Comparison of lipid per-oxidation (MDA nmol/g) levels of tobacco ALDH 3I1 lines under control and 2 weeks cold stress (a); control and 2 weeks drought stress (b); control and 2 week salt stress (c). Data represent mean values \pm SE

MDA levels of wild-type and transgenic plants with the ALDH3I1 gene were measured under control as well as abiotic stress conditions (cold, drought and salt). Under normal growth conditions, the MDA levels of all lines lie in a range of 34 to 39 nmol/g fresh weight of plant. Cold stressed triggered the MDA levels in wild-type plants and it rose to 122 nmol/g FW (4 times of the control). Whereas, the MDA levels of transgenic lines fell in a range of 56 nmol/g (SA3P2 and SA3P12) to 68 nmol/g (SA3P1 and SA3P7). The increase of MDA level is still weak as compared to that in wild-type plants (Fig. 3.26).

The drought stress also had a similar drastic effect on lipid per oxidation. MDA levels of wild-type plants were measured to be 120 nmol/g of fresh weight. The transgenic lines, on the contrary, had not such a high lipid per-oxidation profile. The MDA values for transgenic plants under drought conditions ranged from 58 nmol/g (SA3P12, SA3P13 and SA3P14) to 70 nmol/g (SA3P2).

Salt stress also had a similar catabolic effect on lipids and their per-oxidation in wild-type plants with 120 nmol/g of MDA. On the other hand, the transgenic lines showed improved tolerance to salt stress where the MDA values increased to 79 nmol/g (SA3P7). Line SA3P12 had the lowest level of oxidized lipids (60 nmol/g) among all the lines tested under salt stress.

3.1.21 Proline Content After Drought Stress

The proline content of the plants was measured after 3 days *in vitro* culture of plants on MS medium supplemented with 10% PEG. The results showed that the proline content in the transgenic plants increased after the treatment as compared to the plants grown on MS medium (Fig. 3.27). Whereas in wild-type plants the proline content was decreased after the treatment, an indication of sensitivity of the wild-type plants to PEG induced stress.

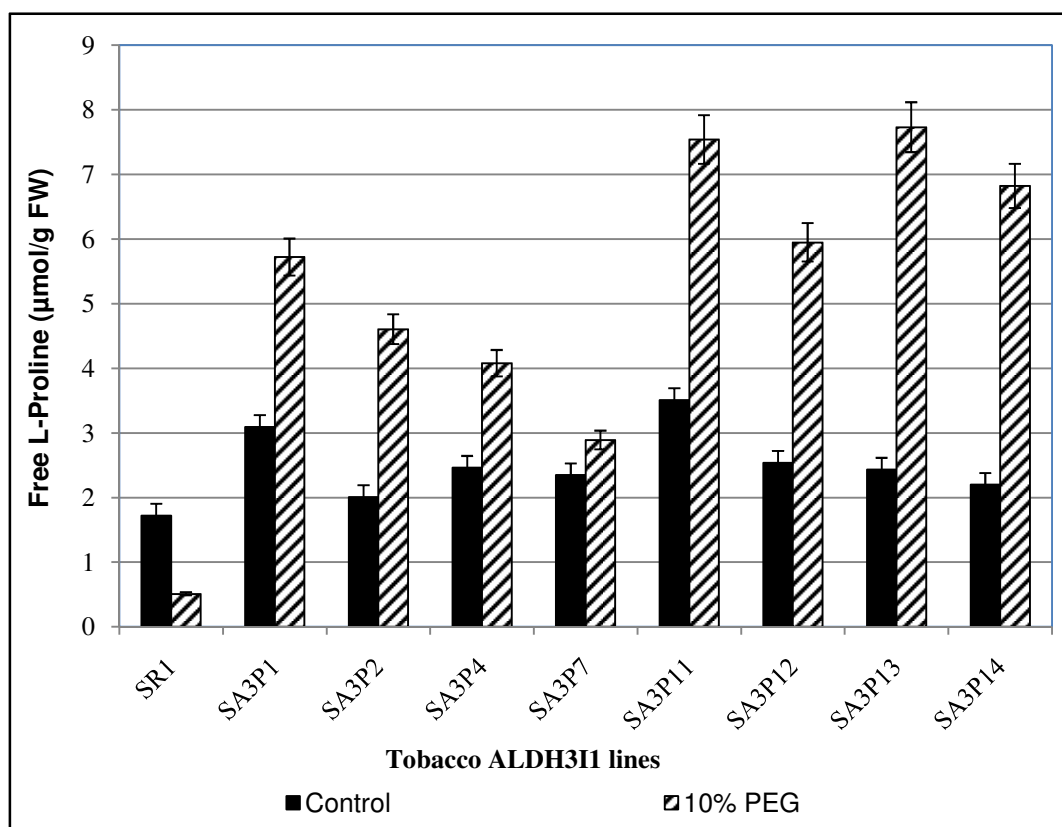


Fig. 3.27: Proline content of the *in vitro* grown plants on 10% PEG medium after 3 days compared to the MS medium (control).

3.2 Part II: Soluble Osmolyte Ectoine

1, 4, 5, 6-Tetrahydro-2-methyl-4-pyrimidinecarboxylic acid (Ectoine) functions as a compatible osmolyte in halophilic bacteria. Ectoine is biosynthesized by three successive enzymatic reactions starting from aspartic β-semialdehyde (ASA). The genes encoding the enzymes involved in the biosynthesis, *ect A*, *ect B* and *ect C*, encoding L-2,4-diaminobutyric acid acetyltransferase (DAT), L-2,4-diaminobutyric acid transaminase (DAA), and L-ectoine synthase (ES), respectively, have been previously cloned in tobacco (Nakayama *et al.*, 2000).

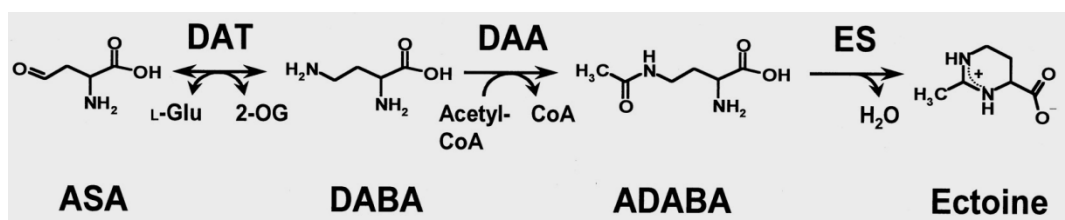


Fig. 3.28: Ectoine biosynthesis pathway. The first step is catalyzed by DAT, which converts L-aspartic-β-semialdehyde (ASA), an intermediate in amino acid metabolism, to L-2,4-diaminobutyric acid (DABA). Then acetylation of DABA results into N⁷-acetyl L-2,4-diaminobutyric acid (ADABA), is promoted by DAA. In the last step, ES catalyzes the cyclic condensation of L-2,4-diaminobutyric acid to yield tetrahydropyrimidine ectoine

3.2.1 Co-bombardment of Tomato

In vitro grown tomato calli, cotyledonary and epicotly ex plants were bombarded with a 1:2:2:2 of vector and *Ect A*, *Ect B* and *Ect C* genes. Some callus grew and lead to few shoot but upon subcultring the shoots to selection media (MS + Kanamycin) the shoots failed to root. Therefore, it was assumed that it had no gene for selectable marker (Kanamycine resistance) and then they were not used further.

3.2.2. Cloning with Point Mutagenesis and PCR Amplification and Agrobacterium mediated transformation

When it was attempted to transform the whole construct in the *Agrobacterium* strain GV3101 or LBA4404 on LB + Kanamycine medium it was unsuccessful many times. Only once a single colony was obtained but it also lacked the *Ect B* gene.



Fig. 3.29: Constructed Ect B + DQ005473 (Ect B cassette) with *PacI* restriction sites in pJET 1.2 shows the cut plasmid DNA with *PacI* from various colonies.

After the creation of the *PacI* sites in EctB + DQ005473 plasmid, it was amplified with PCR and inserted in pJET 1.2 blunt cloning vector. Before using that pJET DNA as insert source for the next cloning step colony PCRs with specific primers and restriction digestions with *PacI* (Fig. 3.29) were done to confirm the presence and orientation of the EctB cassette (3.37 kb in size). All colonies tested proved to be positive.

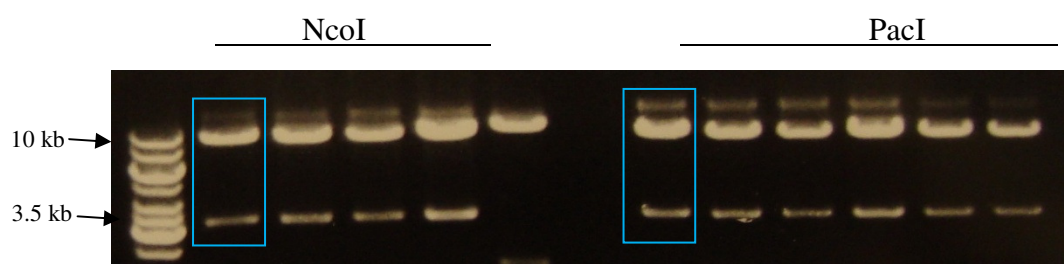


Fig. 3.30: Constructed Ect B cassette in binary vector; pPZP 200 + DQ005456 shows the restriction digestion of the binary vector with EctB cassette with *NcoI* and *PacI* restriction enzymes.

The confirmation of the presence of the EctB cassette was done by restriction digestion with *NcoI* and *PacI* restriction endonucleases. With *NcoI* the required sizes were 3.29 kb and 8.9 kb if in normal orientation and 2.1 kb and 10 kb in the opposite orientation. With *PacI* the expected sizes were 3.37 kb and 8.89 kb. All the colonies tested were found to be positive and the colony highlighted was taken for further cloning due to its sense orientation.

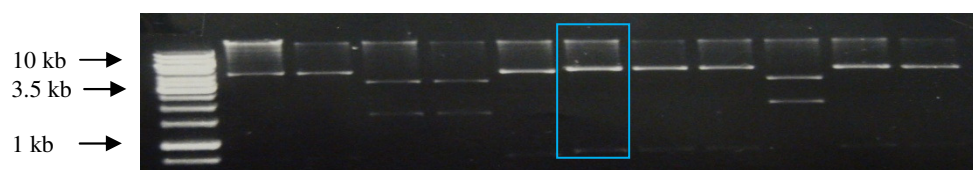


Fig. 3.31: Constructed Ect C+ DQ005453 (Ect C cassette) with *Afl* II restriction sites in pJET 1.2 shows the cut plasmid DNA with *Hind*III restriction enzyme.

After the creation of the *Afl*II sites in EctC + DQ005453 plasmid, it was amplified with PCR and inserted in the pJET 1.2 blunt cloning vector. Before using pJET DNA as insert source for the next cloning step colony PCRs with specific primers and restriction digestions with *Hind*III (Fig.3.31) were done to confirm the presence and orientation of the EctC cassette. The colony highlighted showed the correct 776 bp and 4.39 kb bands and it was further used for cloning.

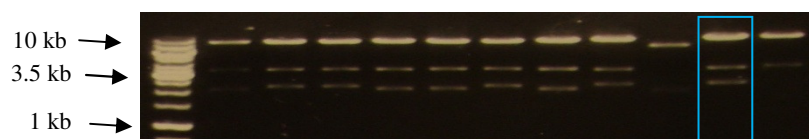


Fig. 3.32: Constructed Ect C cassette + Ect B cassette in binary vector; pPZP 200 + DQ005456 shows the restriction digestion of the binary vector with EctB and Ect C cassette with *Nco*I restriction enzyme.

The confirmation of the presence of the EctB cassette and Ect C cassette was done by restriction digestion with *Nco*I restriction endonuclease (Fig. 3.32). The colony highlighted showed the correct 8.97 kb + 3.55 kb and 1.91 kb bands and it was further used for cloning.

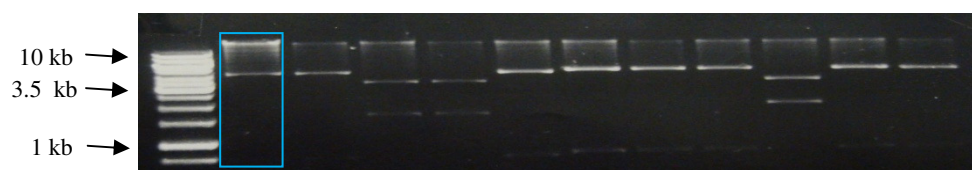


Fig. 3.33: Constructed Ect A + DQ005466 (Ect A cassette) with *Sbf*I restriction sites shows the plasmid DNA cut with *Hind*III.

After the creation of the *Sbf*I sites in EctA + DQ005466 plasmid, it was amplified with PCR and inserted in the pJET 1.2 blunt cloning vector. Before using pJET DNA as insert source for the next cloning step colony PCRs with specific primers and restriction digestions with *Hind*III (Fig.3.33) were done to confirm the presence and orientation of the EctA cassette. The colony highlighted showed the correct 729 bp and 4.11 kb bands and it was further used for cloning.

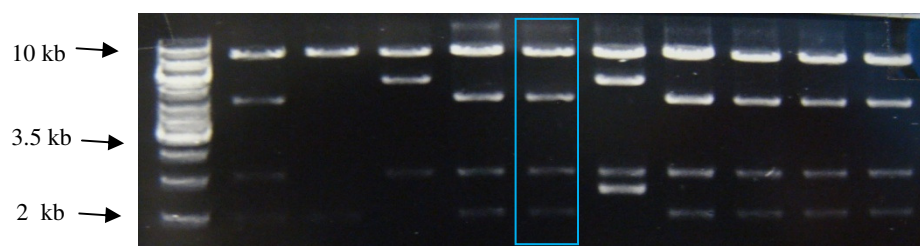


Fig. 3.34: Gel picture showing the restriction digestion of the binary vector containing all the three ectoine genes with *Sal*I.

The confirmation of the presence of the EctA cassette in the binary vector already containing EctB and EctC cassettes was done by digesting the plasmid DNA with *Sal*I restriction endonuclease (Fi. 3.34). The colony highlighted showed the correct 8.29 kb + 4.33 kb + 2.11 kb and 1.54 kb bands and it was further used for cloning.

After getting the final construct, the binary vector with all the three genes, it was transformed first in *Agrobacterium* and then transformed into *Arabidopsis thaliana* wild-type (Col 0) using the floral dip method.

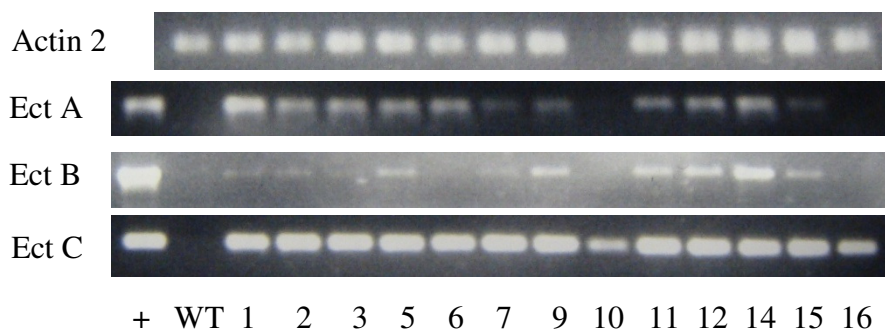


Fig.3.35: RT PCR for Actin2 and Ect A, Ect B and Ect C genes in the transgenic plants containing Ect A, Ect B and Ect C genes. Here in case of positive control (+) with Ect A, Ect B and Ect C genes plasmid DNA is used while in Actin 2 PCRs no plasmid DNA is used. WT= wild-type plants and numbers 1 to 16 are the independent transgenic lines of *Arabidopsis*.

The expression of the ectoine genes was analysed with reverse transcriptase PCR. The transgenic *Arabidopsis* plants showed a variable expression of the three ectoine genes under control growth conditions when determined by RT-PCR. The highest expression was observed in Ect C gene, which was highly expressed in all lines. EctA showed an average expression pattern with a few lines having very little to none expression. The lowest expression was observed for the Ect B gene that was only expressed in a few lines.

4 Discussion

Genetic engineering of plants has become a versatile platform to study gene functions, signaling and improvement of plants. It offers a great potential for genetic manipulation of crops to enhance productivity through increasing resistance to environmental stresses, diseases and pests, and improvement of other qualitative traits. Beyond crop improvement, the ability to engineer transgenic plants is also a powerful and informative means for studying gene function and the regulation of physiological and developmental processes. Many genes that respond to osmotic stress have been described (Kasuga *et al.*, 1999; Saijo *et al.*, 2000; Zhnag *et al.*, 2001). These genes are thought to function not only to protect cells from a water deficit by the production of important metabolic proteins but also to regulate the expression of genes required to mediate signal transduction in response to the stress. This fact makes the genes taking part in signal transduction pathways, especially transcription factor genes, good candidates for crop improvement in stress conditions. Although there is a significant effort in understanding the transcriptional level of signal transduction, the information obtained in this regard is still limited. In this study it was intended to do physiological and molecular characterization of plants which over express two ALDH genes as compared to one gene and genes for the soluble osmolyte, ectoine. The characterization of ALDH genes was previously partially done in *Arabidopsis*. Here few of the double over-expressing lines of ALDH3I1 and ALDH7B4 genes were partially characterized. ALDH genes were also transformed in tobacco in an attempt to see whether their expression pattern under stress conditions is the same in other higher plants (tobacco) in comparison to the model plant *Arabidopsis*. In this study tobacco leaf disc transformation with *Agrobacterium* containing the ALDH genes was carried out. The genes transformed were under the control of the constitutive CaMV35S promoter. At the end of the transformation studies, it took 14 months to get the seeds of the transgenic plants. The long process consisted of callus regeneration, shoot proliferation, elongation and rooting of shoots, transferring the rooted plants in pots, confirmation of the genes via PCR and getting seed from the transgenic plants.

Numerous traits and genes controlling them govern plant stress tolerance. These complex multigenic traits are difficult to establish in crops. Several genes such as barley *HVA1* (Xu *et al.*, 1996), rice *CDPK* (Saijo *et al.*, 2000), alfalfa *Alfin1* (Winicov, 2000), tobacco *NPK1* (Kovtun *et al.*, 2000) and *Brassica GlyI* (Veena *et al.*, 1999) have been ectopically expressed in transgenic plants to enhance stress tolerance. These reports suggest that

transfer of a single gene also can improve stress tolerance in plants (Kasuga *et al.*, 1999; Saijo *et al.*, 2000; Zhnag *et al.*, 2001). Drought pre-treatment and UV-B irradiation revealed that when gene for alfalfa aldose/aldehyde reductase (ALR) enzyme was transferred to tobacco the transgenic plants were more tolerant than wild type SR1 tobacco plants (Hideg *et al.*, 2003).

4.1 Plants Over-expressing ALDH Genes

The double expressing lines of ALDH3I1 and ALDH7B4 were made with the idea to increase the stress tolerance compared to plants which were transferred with only one ALDH gene. Preliminary *in vitro* experiments were performed to select the better performing lines for further characterization. These experiments gave us a quicker insight into the stress tolerance ability of the various lines. Transgenic plants over-expressing either ALDH3I1 or the ALDH7B4 gene were reported to be tolerant to abiotic stress (Kotchoni *et al.*, 2006). These plants over-expressing both genes were made with a hypothesis that they would be more tolerant to stress than their single over expressing single parents. For some of the lines it seemed to be true that they performed better on MS medium with salt (NaCl) and paraquat.

4.1.1 ALDH Transcript Expression

The RT-PCR showed a variable transcript profile among wild-type and transgenic plants. It suggested that expression of ALDH genes was induced by environmental stress and the over-expression of more than one ALDH gene can increase the transcript level compared to wild-type and single over expressing plants. Higher amounts of ALDH genes were reported to be induced under environmental stress than under low oxygen conditions or anoxia (Kuersteiner *et al.*, 2003). ALDH transcripts can express sometimes even in some homologue gene, for instance under salt stress the transcript level of genes homologous to the ALDH7B6 gene start to increase after only five hours of salt or drought stress in salt tolerant rice (*Oryza sativa* L.) cv. Dee-Geo-Woo-Gen plants whereas no ALDH transcript was detected in control plants (Shinozaki *et al.*, 2005).

The transcript accumulation of ALDH3I1 and ALDH7B4 after one week salt and drought stress was checked with gene specific RT-PCR. It showed that ALDH3I1 and ALDH7B4 generally had more transcript accumulation in transgenic lines as compared to wild-type (Col0) Arabidopsis plants under drought stress conditions, as compared to NaCl stress. The presence of higher amounts of ALDH transcripts supports the higher production of ALDH in transgenic plants that leads them to survive better under stress conditions as

compared to non-transgenic plants. Although in wild-type *Arabidopsis* plants the transcript accumulation of ALDH3I1 (Sunkar *et al.*, 2003) and ALDH7B4 (Kotchoni *et al.*, 2006) is reported to start a few hours after stress these studies suggest that co-over-expression of both ALDHs can accumulate higher levels of ALDH transcripts after a prolonged time period.

The expression of more than one transgene does not necessarily lead to better tolerance than with plants over-expressing a single gene. Yang *et al.* (2009) reported that over-expression of three genes resulted only in marginal improvement in the salt tolerance ability of plants as compared to plants over-expressing only one gene. They did find an increase in transcript level in single over expressing transgenic plants as compared to wild-type plants. The transcript expression in our studies is variable. In case of ALDH3I1 it was detected to be higher in single over expressing plants than all the double over expressing lines under drought stress where as under salt stress ALDH3I1 were marginally higher in one of the double over-expressing line suggesting that co-overexpression can lead to higher transcript expression of ALDH genes in plants. The transcript expression of ALDH3I1 in tobacco plants showed no expression in wild-type plants as compared to transgenic plants. The expression of ALDH3I1 transcript was apparently caused by T-DNA insertion. *Arabidopsis* HDG 11 gene has been successfully transformed in tobacco and showed a similar expression pattern and accumulation as in *Arabidopsis* (Yu *et al.*, 2008).

4.1.2 ALDH protein expression

Previous reports on *Arabidopsis*-ALDH gene expression revealed that ALDH3I1 is constitutively and weakly expressed in wild-type plants (Kirch *et al.*, 2001) supports our results in a way that we got the ALDH3I1 gene over-expressed alone (in single over-expression lines) as well as in combination with the over-expression of the ALDH7B4 gene. Accumulation of ALDH3I1 and ALDH7B4 proteins starts as soon as after 8 hrs even in wild-type *Arabidopsis* plants (Kotchoni *et al.*, 2006; Kirch *et al.*, 2005). In the present study it was observed that the accumulation of ALDH3I1 and ALDH7B4 proteins were higher in some double over-expressing lines after a week of stress induction. There were also a few lines that had less protein accumulation than single over-expressing lines. In the present study although expression of ALDH proteins was found in wild-type plants after a week of stress, but it was less than transgenic lines co-over-expressing the two ALDH genes. It suggests that the co-over-expression (or double over-expression) of

ALDH3I1 and ALDH7B4 can lead to production of more ALDH proteins in plants that may help finally to detoxify aldehydes produced under abiotic stress.

The ALDH accumulation profile of the plants under study also revealed variable levels of proteins. Some double over-expressing lines had more ALDH proteins than wild type and single over-expressing plants after stress conditions. The fact that all double over-expressing lines did not produce higher amounts of specific ALDH proteins is supported by the finding of Yang *et al.* (2009) who discovered that over-expression of more than one gene does not necessarily result in higher gene expression than plants over-expressing only one gene. However, it is not always the case, over-expression of ZFHD1 and NACTFs gene leads to the induction of ERD1 (Tran *et al.*, 2006). In addition to this beneficial effect one more effect of co-over-expression was observed normally that the elevated level of ZFHD1 had a negative effect on plant size. The double over-expressing line SA3P6 resulted to be the line with much higher levels of ALDH proteins under stress conditions but no such negative effect was observed at all. Shin *et al.* (2009) suggested that rice ALDH7 is involved in detoxifying various aldehydes formed by oxidative stress during seed desiccation.

Weiser (1970) first proposed that cold hardening might involve changes in gene expression. Later on, cycloheximide had been shown to inhibit cold acclimatization in potato (Chen *et al.*, 1993) and wheat (Turnova, 1982). Numerous other studies had indicated that changes in protein composition occur at low temperatures (Uemura and Yoshida, 1984; Guy *et al.*, 1987; Mohapatra *et al.*, 1987; Roberston *et al.*, 1987). Further, *in vitro* translation of poly (A+) RNA isolated from control and cold-treated spinach (Guy *et al.*, 1985), rapeseed (Meza-Basso *et al.*, 1986), and alfalfa (Mohapatra *et al.*, 1987) has indicated that specific mRNAs accumulate at low temperatures while others decrease. Over-expression of the dehydration responsive element gene (DREB1A) improved drought and low temperature stress tolerance in tobacco. The stress inducible promoter rd29A minimized the negative effects on plant growth in tobacco (Kasuga *et al.*, 2004).

4.1.3 Prolonged Drought and Salt Stress

The prolonged drought stress clearly screened out the double over-expressing lines, line SA37P10 was the most tolerant line and SA37P44 plants also had much higher survival than wild type and single over-expressing lines (Fig. 3.10). These results suggest that there is no negative effect of the over-expression of the two ALDH genes in Arabidopsis rather they resulted in a higher tolerance level to drought. It has been reported that co-

expression is something beneficial and can even control the negative phenotypic effects (Tran *et al.*, 2006). ALDH3I1 and ALDH7B4 also resulted to be such genes that have no negative effect on each other and in some lines it rather can produce more stress tolerant plants and that effect is more than the over-expression of a single gene alone. Over expression of an alfalfa ALR gene in tobacco improved tolerance against drought (Hideg *et al.*, 2003). Activated expression of an Arabidopsis HD-START protein conferred drought tolerance with improved root system (Yu *et al.*, 2008). These findings support our findings that longer roots were observed after salt stress of transgenic tobacco plants with ALDH3I1 gene.

4.1.4 Lipid Peroxidation

ALDHs have been reported for an *A. thaliana* mitochondrial succinic-semi ALDH (SSADH), which is involved in γ -aminobutyric acid (GABA) metabolism and belongs to family 5 of *A. thaliana* ALDH (*ALDH5F1*). SSADH was reported to reduce levels of reactive oxygen intermediates by supplying NADH and succinate under conditions that inhibit the tricarboxylic acid (TCA) cycle and impair respiration (Bouché *et al.*, 2003). A T-DNA insertion mutagenesis showed the potential of SSADH in protecting *A. thaliana* plants against excessive accumulation of ROS under abiotic stress. Null SSADH mutant plants accumulate higher amounts of ROS in leaves during exposure to light when compared to wild-type plants (Bouché *et al.*, 2003).

Lipid per-oxidation was measured by two different methods. An older method (Heath and Packer, 1968) used the lipid per-oxidation at only two wave-lengths whereas an improved method (using measurements at three wave-lengths) suggested by Hodges *et al.* (1999) proved to give a more accurate measure of MDA levels. The method of Hodges *et al.* (1999) led us to find accurate levels of MDA as they exclude the carbohydrates and anthocyanins to be measured together with MDA. It has been reported that MDA levels in wild-type Arabidopsis plants increased with salt stress (NaCl or KCl) but the comparative increase in MDA levels of plants over-expressing either ALDH3I1 or ALDH7B4 was less than in wild-type (Kotchoni *et al.*, 2006). Our results are supported by these findings which confirm the crucial role of ALDH3I1 and ALDH7B4 in detoxification of reactive aldehydes produced from lipid per-oxidation. But the MDA levels of plants over-expressing both ALDH3I1 and ALDH7B4 was only marginally lower than single over-expressing plants. Although ALDH3I1 is localized in chloroplasts and ALDH7B4 in the cytoplasm (Kotchoni *et al.*, 2006), it cannot be explained why over expression of both

genes together did not always result in improved physiology of all these double over expressing plants. It has been suggested that over expression of more than two genes was not beneficial as expected might be due to the differential over-expression localization in specific tissues (Yang *et al.*, 2009). Low temperature resulted in high MDA in SR1 as compared to the tobacco plants over-expressing alfalfa ferritin or aldose reductase (Hegedus *et al.*, 2002). Mano *et al.* (2005) discovered that in addition to lipid-peroxidized aldehydes, the reactive carbonyls (aldehydes and ketones that have α and β -unsaturated bond) are produced in plants and the over-expression of At-AER genes lead to scavenge them too. Our results are in accordance with Li *et al.* (2007) who reported a fourfold increase in MDA levels in wild-type tobacco, whereas in transgenic plants over-expressing 1-fructosyltransferase (1-SST) genes that increase was not as high as in wild-type.

4.1.5 Photosynthetic parameters: Gas Exchange

Keeping in mind the deleterious effects of reactive aldehydes on lipids and membranes, it seemed logical to measure the activity of the chloroplast, one of the most important membranous organelle in plants. For this purpose a recent device, GFS 3000, designed by WALZ, Germany was used. The wild-type plants showed less gas exchange resulting from photosynthesis than transgenic plants under either salt or drought stress. This supports the hypothesis that less chlorophyll damage had occurred because the lipids binding its membrane were protected due to more production of ALDHs. In some double over-expressing lines the difference in CO₂ gas exchange is higher at high light intensities, suggesting that at high light intensity the input light energy is higher and leads to higher gas exchange only in plants where chlorophyll is not damaged or less damaged. The variation may also be attributed to defective light receptors or altered stomatal movements due to stress (Eckert and Kaldenhoff, 2000). Abiotic stress normally decreases the maximum efficiency of PSII of plant leaves (Meyer *et al.*, 1997; Degl'Innocenti *et al.*, 2003; Calatayud *et al.*, 2006) resulting in an increased fraction of closed PSII reaction centre and an enhancement of dissipation of excess excitation energy via non-radioactive mechanisms due to an inhibition of CO₂ fixation (Guidi *et al.*, 1997). Our findings are also supported by the results of Xu *et al.* (2007), who found reduced photosynthesis in drought affected wheat. The over expression of binding proteins (BIP) of endoplasmic reticulum in tobacco revealed that these binding proteins act as protectants under water stress. Binding proteins over expression led to maintenance of

shoot turgidity and water content and also resulted in higher CO₂ assimilation compared to wild-type tobacco plants (Alvim *et al.*, 2001).

4.1.6 Chlorophyll content

Chlorophyll is the green pigment of the plants that is the source of energy capture and then later produces energy for plants themselves. Under stress conditions the damage of this membranous structure results in the inability of plants to conduct photosynthesis. The chlorophyll measurements from plants under methyl viologen stress disclose that in a few double over expressing lines the chlorophyll content was higher than in wild-type and single over-expressing line. The line SA37P44 showed an increase in chlorophyll level under stress conditions compared to normal growth conditions. These findings confirm the reports that over-expression of ALDH leads to higher chlorophyll content in transgenic plants under stress conditions (Kotchoni *et al.*, 2006). Our results are also supported by the findings of Sunkar *et al.* (2003) who also found higher chlorophyll content in transgenic plants under methyl viologen stress due to over-expression of ALDH3I1 (formerly called At-ALDH3; new nomenclature by Kirch *et al.*, 2004). Transgenic lines with CaMVF9 and ALR1/9 lines had a chlorophyll content higher than wild-type tobacco SR1 (Hegedus *et al.*, 2002) under cold stress. Pea DNA helicase 45 (PDH45) was over-expressed in tobacco and showed increased tolerance to salinity stress indicated by the presence of higher chlorophyll content and retention of protein in the leaf discs of salt stressed transgenic plants whereas wild-type leaves turned yellow and had less protein. They reported also decreasing chlorophyll content with increasing NaCl (0, 100, 200, 300 mM) in wild-type that was less compared to transgenic tobacco plants over-expressing the PDH45 gene (Sanan-Mishra *et al.*, 2005). Similarly, when iron binding protein, ferritin was over expressed in tobacco plants the transgenic plants had higher chlorophyll content under 10µM paraquat than wild-type plants (Deak *et al.*, 1999). Additionally, transgenic plants had higher Fv/Fm ratio under 10µM paraquat with the same genes.

4.1.7 Chlorophyll Fluorescence

In the present studies higher chlorophyll fluorescence (Fv/Fm) in transgenic plants under stress conditions are supported by the observations that under low temperature stress wild-type plants of tobacco (SRI) showed lower Fv/Fm ratios as compared to Alfalfa ferritin overproducing CaMVF9 transgenic tobacco and alfalfa aldose reductase over-expressing ALR1/9 lines (Hegedus *et al.*, 2002). With respect to drought, the negative

impact on photosynthesis is well-documented, with carbon assimilation declining progressively with increasing water discrepancy as a result of both stomatal and metabolic limitations (Chaves, 1991; Tezara *et al.*, 1999; Flexas and Medrano, 2002; Cornic, 2000). Thus, non-invasive measurement of photosynthesis by chlorophyll *a* fluorometry (Oxborough, 2004; Baker, 2008) may possibly offer a means to establish plant viability and performance in response to drought. The chlorophyll fluorescence values for our studies showed a dramatic decrease after abiotic stress treatment in wild-type plants, suggesting the improper function of chlorophyll in the wild-type plants. These results are in accordance with the findings that Fv/Fm ratio was reduced to more than half under cold stress and almost tenfold decreased under drought stress in wild-type as compared to transgenic tobacco with 35S: DREB1A gene (Kasuga *et al.*, 2004). Similarly, the PS II activity (Fv/Fm) appeared to be less decreased in transgenic plants over expressing At-AER genes in tobacco Mano *et al.* (2005).

Measurement of chlorophyll fluorescence by probe-based systems has been utilized for non-invasive analyses of stress-induced perturbations of photosynthesis for several decades. Indeed, dissection and analysis of the brisk polyphasic chlorophyll *a* fluorescence transient OJIP (Lazar, 2006), a technique applied previously to measure tolerance to light (Oukarroum and Strasser, 2004) and chilling (Strauss *et al.*, 2006) stresses, was recently employed to assess the response of several barley cultivars to non-lethal drought stress (Oukarroum *et al.*, 2007). The introduction of chlorophyll fluorescence imaging systems has allowed acquisition of fluorescence data from larger sample areas than probe-based systems (Omasa *et al.*, 1987; Quilliam *et al.*, 2006), thereby enabling simultaneous measurement of several specimens and the identification of spatial heterogeneities in photosynthesis across whole leaves or rosettes. Chlorophyll fluorescence techniques have also been successfully utilized to examine the impact of numerous environmental stresses (Baker and Rosenqvist, 2004), including cold (Ehlert and Hinch 2008), high light (Muller-Moule *et al.*, 2004) and wounding (Quilliam *et al.*, 2006).

4.1.8 Proline accumulation

Amino acids can be used by higher plants as osmolytes and as a source of nitrogen and carbon. They can be translocated from other parts of the plant to stress-affected organs or released by the hydrolytic degradation of protein. The products of protein hydrolysis, such as proline, glutamic acid, and aspartic acid, can be used in *de novo* protein synthesis

or as osmoprotectants. Among the amino acids proline is the most effective against salt stress (Maggio *et al.*, 2002). Proline is an osmoprotectant that is widely distributed among organisms. This osmoprotectant is an organic solute frequently synthesized in crop plants under salt and water stress (Miller *et al.*, 2005). It is widely believed to function as a protector or stabilizer of enzymes or membrane structures that are sensitive to dehydration or ionically induced damage (Reddy *et al.*, 2004).

It has been suggested that proline protects plant tissues against osmotic stress because it is an osmo-solute, a source of nitrogen, a protectant for enzymes and cellular structures (Le-Rudulier *et al.*, 1984), and a scavenger for hydroxyl radicals (Smirnoff and Cumbes, 1989). In addition to these, proline accumulation in plants may function in the storage of energy, and reducing power and amino nitrogen storage that would be of crucial importance in the maintenance of repair processes operative after osmotic stress and in the rapid restoration of cellular homeostasis (Verbruggen *et al.*, 1996). Proline levels of salt (NaCl) treated plants reached a peak after 62h and remained the same till 96h. However, glycine betaine reached a peak after 96h of NaCl treatment (Goas *et al.*, 1982) in half strength Hoagland's medium in Aster plants. Our results showed a similar pattern; in the transgenic plants containing the ALDH3I1 gene the proline levels were increased after stress treatment but were decreased in wild-type plants. The accumulation of proline in transgenic tobacco plants induced by some other genes responsible for the proline production (D-pyrroline-5-carboxylate synthetase; P5CS) also leads to the tolerance of plants to abiotic stress (Hong *et al.*, 2000). It has been reported that overproduction of proline also enhanced root biomass and flower development in transgenic plants under drought-stress conditions (Kishore *et al.*, 1995). These findings support our results and we observed an increase in plant biomass under salt and drought conditions that resulted in increased tolerance of transgenic plants under abiotic stress compared to wild type plants. It was found that intermediates of proline biosynthesis and catabolism such as glutamine and P5C could increase the expression of several osmotically regulated genes in rice such as salT and the dehydrin dh4 (Iyer and Caplan, 1998).

4.1.9 ROS and cell death

In the present study the leaf discs of wild-type tobacco showed H₂O₂ production that increased with the increasing concentration of paraquat solution. These results are in line with the report of Price and Hendry (1991), who found a positive correlation between the susceptibility to drought and ROS production during the stress in various grasses and

cereals. Transgenic R1C-Prx (Rice 1Cys-peroxiredoxin) tobacco plants tolerated 5 mM H_2O_2 solutions when radicals stress was measured after infiltrating whole plants that resulted in lesions on fewer leaves as compared to wild type plants (Lee *et al.*, 2000). Exogenous applications of salisalic acid can also lead to improved chilling tolerance and resulted in decreased levels of H_2O_2 production (Kang *et al.*, 2003) in banana seedlings. The over-expression of At-AER led to a reduced area of necrosis as observed in tobacco plants over expressing At-AER that tolerated oxidative stress but only to a mild level of (0.2 μM) MV in the dark but higher concentration of MV lead to no tolerance Mano *et al.* (2005). Drought stressed SR1 tobacco plants had higher H_2O_2 levels as compared to plants over expressing alfalfa ALR gene (Hideg *et al.*, 2003).

Under abiotic stress conditions such as those of photooxidative stress, highly reactive species, such as perhydroxyl radical [HO_2], H_2O_2 (the protonated form of superoxide radical), or singlet oxygen, would oxidize both membrane lipids and free fatty acids to produce various species of lipid peroxides, from which various reactive carbonyls can be generated through non-enzymatic reactions (Lee *et al.*, 2001; Schneider *et al.*, 2001). It is also possible that acrolein, the smallest and one of the most toxic 2-alkenals, is produced during the oxidation of lipids (Uchida *et al.*, 1998). When reactive carbonyls are produced continuously, glutathione (GSH), the primary defense molecule against reactive carbonyls in cells, is consumed, leading to an exacerbation of oxidative stress (Uchida, 2003; Park *et al.*, 2005).

Cell death was found almost exclusively in the palisade parenchyma peri-veinal region and, thus, shares spatial location with cell death induced by ozone exposure (Pellinen *et al.*, 1999; Rao *et al.*, 2000b; Schraudner *et al.*, 1998), and by some biotic interactions (Alvarez *et al.*, 1998; Tamagnone *et al.*, 1998; Tang *et al.*, 1999b). This particular cell death pattern could be attributed directly to changes in H_2O_2 homeostasis in the tissue because the high H_2O_2 production and subsequent cell death in CAT1AS plants were both inhibited by exogenously applied catalase and could be mimicked by H_2O_2 generating compounds (Dat *et al.*, 2003). The containment of cell death in the palisade parenchyma layer may be explained by higher photo respiratory activities and/or lower antioxidant capacity in this cell layer, both facilitating the accumulation of the threshold H_2O_2 levels needed to activate an irreversible cell death programme. There is another possibility that additional factors needed to induce or execute cell death are predominantly present in palisade cells (i.e. lipid peroxidation products and other cellular messengers, such as NO

or ROS), which is supported by the recently proposed close interplay between H_2O_2 , O_2^- , and NO during the initiation of cell death (Delledonne *et al.*, 2001; Torres *et al.*, 2002).

4.1.10 Electrolyte Leakage

Assessing membrane stability by measuring electrolyte leakage (%) is a very good indicator of stress tolerance in plants and is successfully used for testing freeze (Campos *et al.*, 2003; Nunes and Smith, 2003), drought (Phillips *et al.*, 2008) and salt tolerance (Stevens *et al.*, 2006; Lopez-Perez *et al.*, 2009). Our results are in accordance with Li *et al.* (2007) who also observed a high increase (70%) of electrolyte leakage in wild type tobacco as compared to transgenic (31%) plants over-expressing sucrose 1-fructosyltransferase (1-SST) genes under freezing stress. Three fold higher ion leakage percent was observed in wild type tobacco plants as compared to transgenic tobacco with 35S: DREB1A genes under drought conditions (Kasuga *et al.*, 2004). Four fold higher ion leakage percent was detected in wild type tobacco plants as compared to transgenic tobacco with 35S: DREB1A genes under cold conditions (Kasuga *et al.*, 2004). Under oxidative stress the over-expression of At-AER resulted in reduced ion leakage in tobacco Mano *et al.* (2005). However, no effect on electrolyte leakage was observed in SR1 and transgenic tobacco lines over-expressing alfalfa ferritin or aldose reductase (Hegedus *et al.*, 2002).

In addition to ALDH two other groups of plant enzymes that scavenge the lipid peroxide-derived aldehydes, i.e. AER (Mano *et al.*, 2001, 2002, 2005) and ALR (Hideg *et al.*, 2003), have distinct substrate specificities and hence appear to act complementary in scavenging lipid peroxide derived toxins in plants. At-AER is reported to be highly specific to 2-alkenals including HNE and to oxenes (Mano *et al.*, 2005) but unreactive with n-alkanals (Mano *et al.*, 2002). Arabidopsis ALDH3 is highly reactive with n-alkanals but not with HNE (Kirch *et al.*, 2001). Thus, 2-alkenals such as HNE and HHE are preferentially reduced by AER to form alkanals. Although less toxic than 2-alkenals, alkanals are still reactive because of the aldehyde mobility and will be oxidized with ALDH to inert carboxylates. Alternatively, aldehydes may be reduced to alcohols with ALR (Oberschall *et al.*, 2000).

Jaglo *et al.* (2001) showed that constitutive over-expression of the *Arabidopsis* C-repeat/dehydration responsive element binding factor (CBF) genes in transgenic *Brassica napus* plants induced expression of orthologs of *Arabidopsis* CBF-targeted genes and increased the freezing tolerance of plants. Hsieh *et al.* (Hsieh *et al.*, 2002a; Hsieh *et al.*,

2002b) also reported that tomato plants ectopically expressing *Arabidopsis* CBF1/DREB1B showed enhanced resistance to water deficit, chilling and oxidative stresses. These results indicate that the CBF/DREB gene can be used to improve the multi-stress tolerance of agriculturally important crops by gene transfer. However, transgenic *Arabidopsis* overexpressing DREB1A caused severe growth retardation under normal growth conditions. Transgenic tomato overexpressing CBF1 also showed the dwarf phenotype under unstressed normal growth conditions (Hsieh *et al.*, 2002a; Hsieh *et al.*, 2002b). Use of the stress-inducible *rd29A* promoter instead of the constitutive 35S CaMV promoter for the overexpression of DREB1A in transgenic *Arabidopsis* minimizes the negative effects on plant growth (Kasuga *et al.*, 1999).

4.2 Soluble Osmolyte Ectoine

Although attempts have been made to metabolically engineer plants for abiotic stress tolerance but the success is still limited due to the low level of osmolytes produced. In plants that naturally accumulate osmoprotectants, the levels are typically 5–50 $\mu\text{mol g}^{-1}$ fresh weight (6–60 mM on a plant water basis) and are highest during exposure to osmotic stress since accumulation is usually to some extent stress-induced (Rhodes and Hanson, 1993; Bohnert *et al.*, 1995). In plant cells, osmoprotectants are typically confined mainly to the cytosol, chloroplasts, and other cytoplasmic compartments that together occupy 20% or less of the volume of mature cells (the other 80% is the large central vacuole). Natural osmoprotectant concentrations in cytoplasmic compartments can therefore reach or exceed 200 mM. Such concentrations are osmotically significant and so have pivotal roles in maintaining cell turgor and the driving gradient for water uptake under stress (Rhodes and Samaras, 1994). It has been reported that drought tolerance in transgenic tobacco is improved by genetically engineering the synthesis of trehalose or fructan (Pilon-Smits *et al.*, 1995; Holmström *et al.*, 1996; Romero *et al.*, 1997). However, the concentrations of these sugars in transgenic plants were too low to confer a conventional osmolyte effect, which engineered mannitol and proline have been shown to confer (Tarczynski *et al.*, 1993; Kishor *et al.*, 1995). Crowe *et al.* (1984) described that the interaction between trehalose and dipalmitoyl phosphatidylcholine was an important factor in the ability of trehalose to stabilize the dry membranes in anhydrobiotic organisms. The water-stress protective effect of fructans was induced by membrane-fructan interaction (Demel *et al.*, 1998). These reports suggested that a low-level

accumulation of trehalose or fructan contributed to protect the functions of the cell membrane in transgenic plants rather than conferring an osmolyte effect.

The transformation of genes responsible for ectoine biosynthesis had always been problematic. It has a complex nature of expression probably due to the various intermediate compounds out of which N^γ-acetyl L-2, 4-diaminobutyric acid (ADABA) is reported to be lethal in plants if over-expressed alone, possibly due to conversion of ADABA to 4-diaminobutyric acid, GABA (Bohnert *et al.*, 2003). We even observed some toxicity at the bacterial level. When the whole construct was transformed in the *Agrobacterium* strain GV3101 or LBA4404 on LB (+ Kanamycine) medium it was unsuccessful many times. Only once a single colony was developed but it also lacked the EctB gene. This observation supports the finding of Bohnert *et al.*, (2003) that over expression of EctB gene is toxic due to the accumulation of GABA. The fact that only one colony developed, even that lacked the EctB gene, leads to the conclusion that GABA production is toxic to *agrobacterium* and only one colony was able to knock out the EctB gene in it while keeping the EctA and EctC genes. When the three genes responsible for the soluble osmolyte ectoine were over-expressed in tobacco plants, the salinity tolerance was enhanced. This could be explained in two ways. Firstly, maintenance of root function was improved so that water can be taken up consistently and supplied to shoots under saline conditions. Secondly, the nitrogen supply to leaves was enhanced by increasing transpiration and by protecting the rubisco proteins from deleterious effects of salt, thereby improving the rate of photosynthesis (Moghaieb *et al.*, 2006).

The three ectoine genes showed a varied expression pattern in our studies. The presence of all the three genes was confirmed via genomic PCRs. However, EctB gene was not expressed in all the transgenic plants. These results can propose a hypothesis that there may be some internal defense mechanism of *Arabidopsis* that suppressed the expression of EctB (less production of the toxic ADABA/GABA) under normal growth conditions or it may only be expressed under salinity. Moghaieb *et al.* (2006) detected ectoine levels in stressed transgenic plants up to twofold higher in roots than in leaves. However, the maximum level of ectoine detected was only 55.5 $\mu\text{mol/g}$ fresh weights of roots.

The low expression of EctB gene can also be due to the limitation posed by restricted availability of aspartate semialdehyde (ASA) which is the metabolic precursor, that is supplied by the plant for biosynthesis of ectoine (Rai *et al.*, 2002). We get low ectoine level in transgenic plants under normal growth conditions. In plants ectoine levels detected were only in a range of 10-250 nM/g fresh weight while in bacteria ectoine

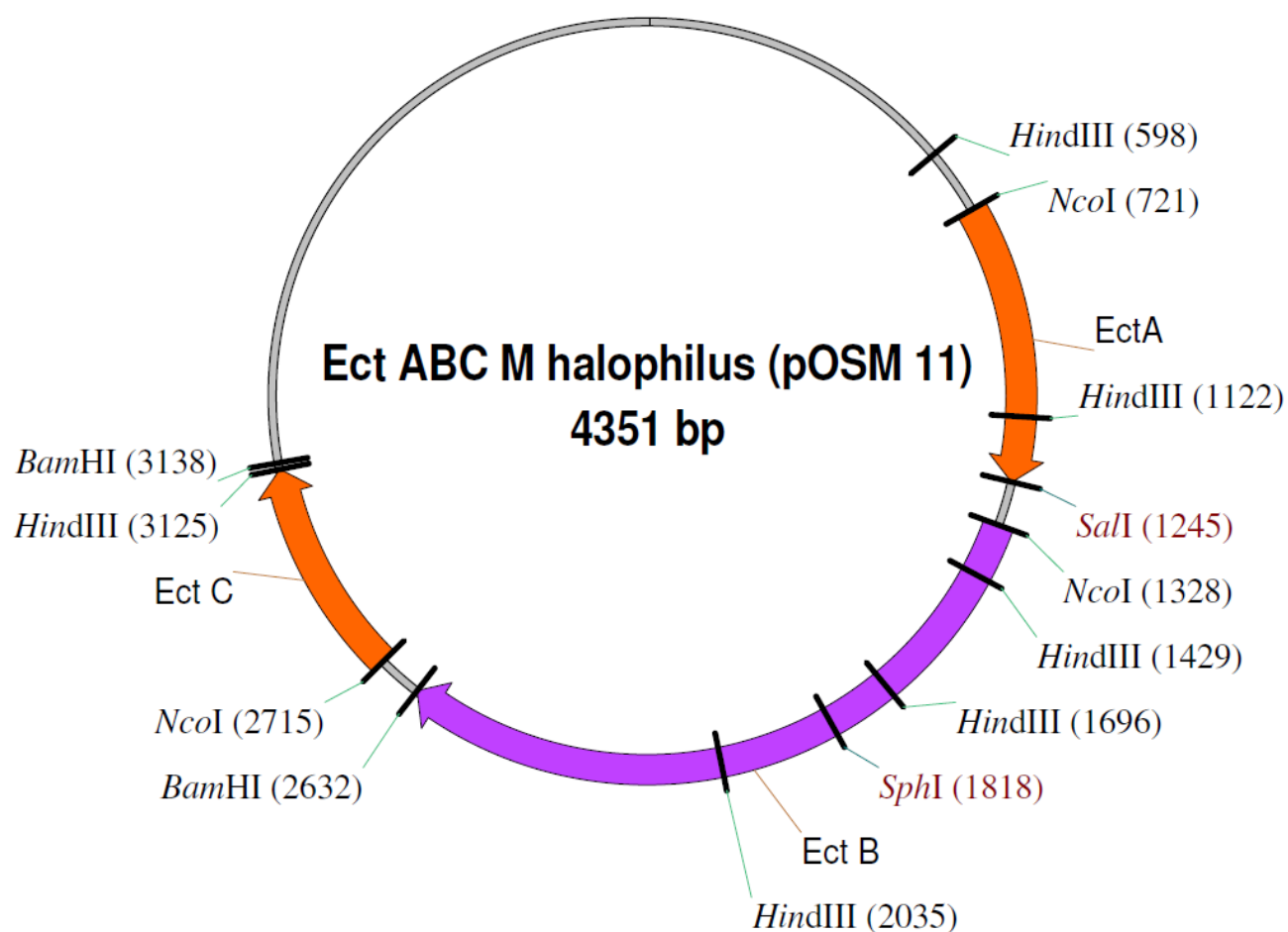
produced can be up to 2.5 g/L of the culture volume (Nagata *et al.*, 2008). Possible explanations for the low ectoine levels in plants include: (a) poor translation of one or more of the bacterial sequences due to divergent codon preferences in bacteria and plants; (b) low availability of the aspartate semialdehyde and acetyl-CoA substrates in the cytosol where the ectoine enzymes were presumably expressed, since these intermediates may be synthesized and used chiefly in chloroplasts (Nawrath *et al.*, 1994; Galili, 1995); and (c) degradation of ectoine. These three problems; codon bias, compartmentation, and catabolism, are recurrent ones in plant metabolic engineering.

5 Zusammenfassung

Das Wachstum von Pflanzen sowie deren Ertrag kann durch eine Vielzahl abiotischer Stressfaktoren beeinträchtigt werden. Diese Stressoren beeinflussen physiologische und metabolische Stoffwechselwege was wiederum zur Entstehung einer Reihe schädlicher Substanzen wie etwa Aldehyden oder reaktiven Sauerstoffspezies führen kann. Die Proteinfamilie der Aldehyd Dehydrogenasen (ALDHs) spielt in vielen Pflanzen eine wichtige Rolle in der Beseitigung dieser potentiell toxischen Verbindungen. Aldehyde sind zwar auch wichtige Zwischenstufen in zahlreichen biochemischen Stoffwechselwegen, können aber durch den Einfluss von Salz-, Trocken-, Hitze- und Kältestress in für die Zelle toxischen Mengen akkumulieren. Im Rahmen der vorliegenden Arbeit wurden transgene Arabidopsis Pflanzen, welche Überexpressionskonstrukte für ALDH3I1 und ALDH7B4 tragen, im Hinblick auf eine veränderte Stresstoleranz untersucht. Dazu wurden RNA- und Proteinextrakte hergestellt und als Ausgangsmaterial für RT-PCR und Western Blots verwendet, um die Integration und Expression der transgen Konstrukte zu verifizieren. In Western Blot und RT-PCR Analysen zeigte sich, dass die mit Salz (200 mM NaCl) und Trockenstress behandelten transgenen Pflanzen eine gegenüber dem Wildtyp gesteigerte Translation bzw. Transkription der beiden ALDHs 3I1 und 7B4 aufweisen. Darüber hinaus wurden Veränderungen auf physiologischer Ebene durch Messung des Gasaustausches sowie Quantifizierung der in den Zellen stattfindenden LipidPeroxidation ermittelt. Die transformierten Pflanzen zeigten dabei sowohl unter Trocken- als auch Salzstress eine im Hinblick auf den Wildtyp gesteigerte CO₂ Assimilation, die mit einer erhöhten Photosynthetischen Aktivität gleichzusetzen ist. Ebenso war die LipidPeroxidation in Wildtyp Pflanzen unter Stresseinfluss im Vergleich mit den Überexpressions-Pflanzen unter den gleichen Bedingungen stärker erhöht. Um diese in *A. thaliana* erhaltenen Ergebnisse zu bestätigen, wurden die betreffenden ALDH Überexpressionskonstrukte auch zur Transformation von Tabakpflanzen herangezogen. Die so erhaltenen transgenen Pflanzen wurden ebenfalls im direkten Vergleich mit der Wildtyp Variante auf eine gesteigerte Toleranz unter Stressbedingungen getestet. Die durch einen konstitutiven oder Stress induzierbaren Promotor erreichte Überexpression von ALDH3I1 und ALDH7B4 in den transgenen Tabakpflanzen, verbesserte die Toleranz gegenüber oxidativem-, Salz- und Trockenstress, was sich in einem gesteigertem Wachstum unter den genannten Bedingungen widerspiegelte. Darüber hinaus konnte gezeigt werden, dass die transgenen Pflanzen unter Stressbedingungen eine geringere Menge reaktiver Sauerstoffspezies

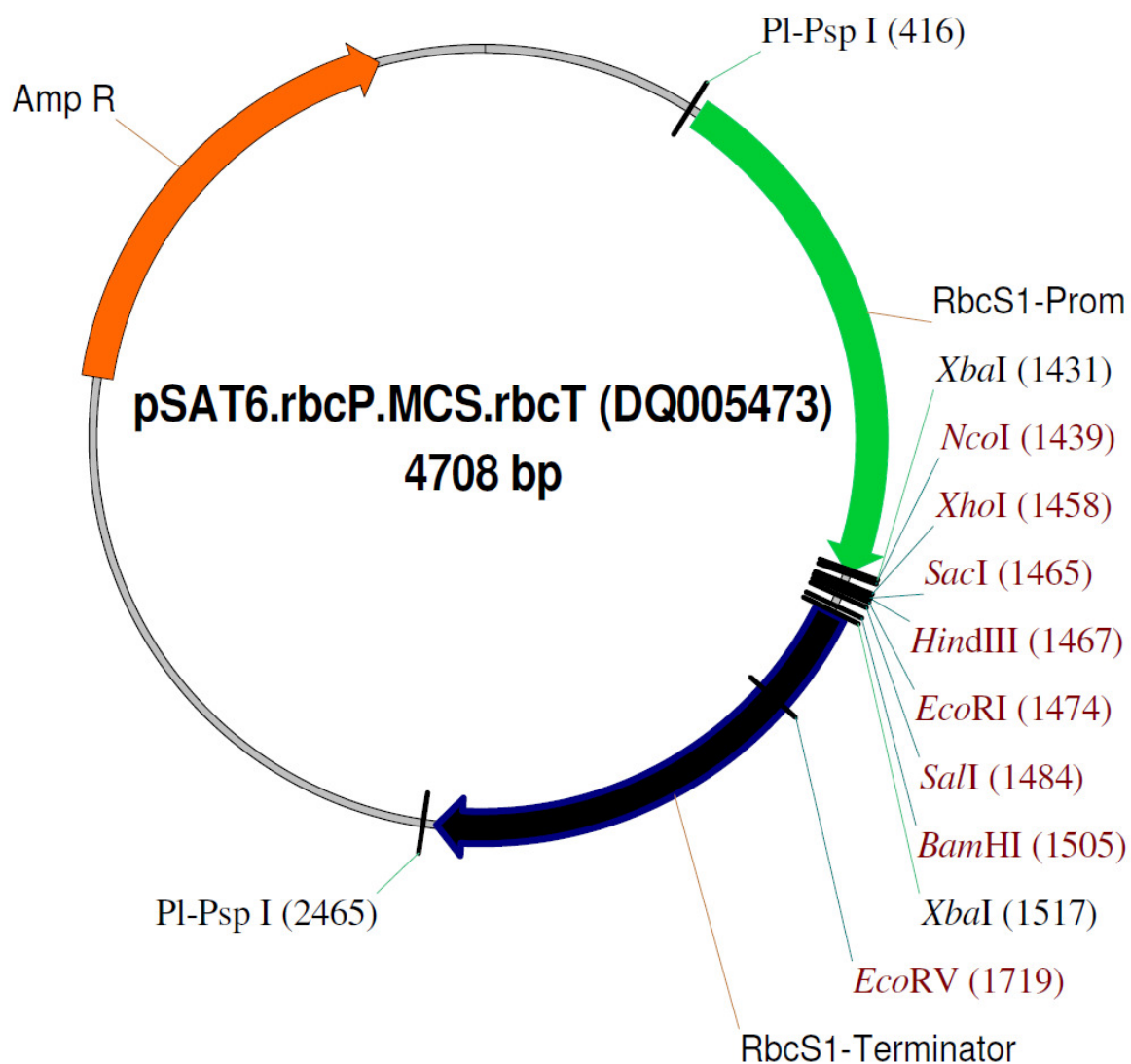
akkumulieren, was gleichbedeutend mit einer reduzierten Schädigung zellulärer Strukturen, insbesondere der Zellmembran, ist. Ein ähnliches Bild zeigte sich beim Chlorophyllgehalt, was auf eine/n gesteigerte Photosyntheserate/Gasaustausch schließen lässt. Malondialdehyd, welches ein Endprodukt der Lipidperoxidation ist, wurde unter Stressbedingungen in den transgenen Tabakpflanzen im Vergleich zum Wildtyp ebenfalls in geringeren Mengen festgestellt. Weiterhin wurden unter Trockenstress höhere Mengen Prolin in den transformierten Pflanzen akkumuliert, als dies im Wildtyp der Fall war. Die Untersuchungen suggerieren, dass die Überexpression der *A. thaliana* ALDHs in Tabak, diesem eine gesteigerte Stresstoleranz verleihen. Zusätzlich zu den ALDH orientierten Experimenten wurden in einem zweiten Projekt alle drei für die Synthese des kompatiblen Soluts Ectoin (1, 4, 5, 6- tetrahydro-2-methyl-4-pyrimidincarbonsäure) benötigten Gene aus dem halophilen Bakterium *Marinococcus halophilus* unter Kontrolle individueller Promotoren bzw. Terminatoren in einen binären Vektor kloniert. Dieses Konstrukt wurde daraufhin in *Arabidopsis thaliana* transformiert und selektierte Transformanten wurden auf die Expression des kompatiblen Soluts hin überprüft. Es konnte festgestellt werden, dass die einzelnen Ectoin Gene in unterschiedlichen Linien in variabler Stärke transkribiert werden und das Pflanzen die alle drei Ectoinsynthesegene enthielten unter normalen Bedingungen, wenn auch in geringem Maße, Ectoin gebildet hatten.

6 Maps of Plasmid vector used and created

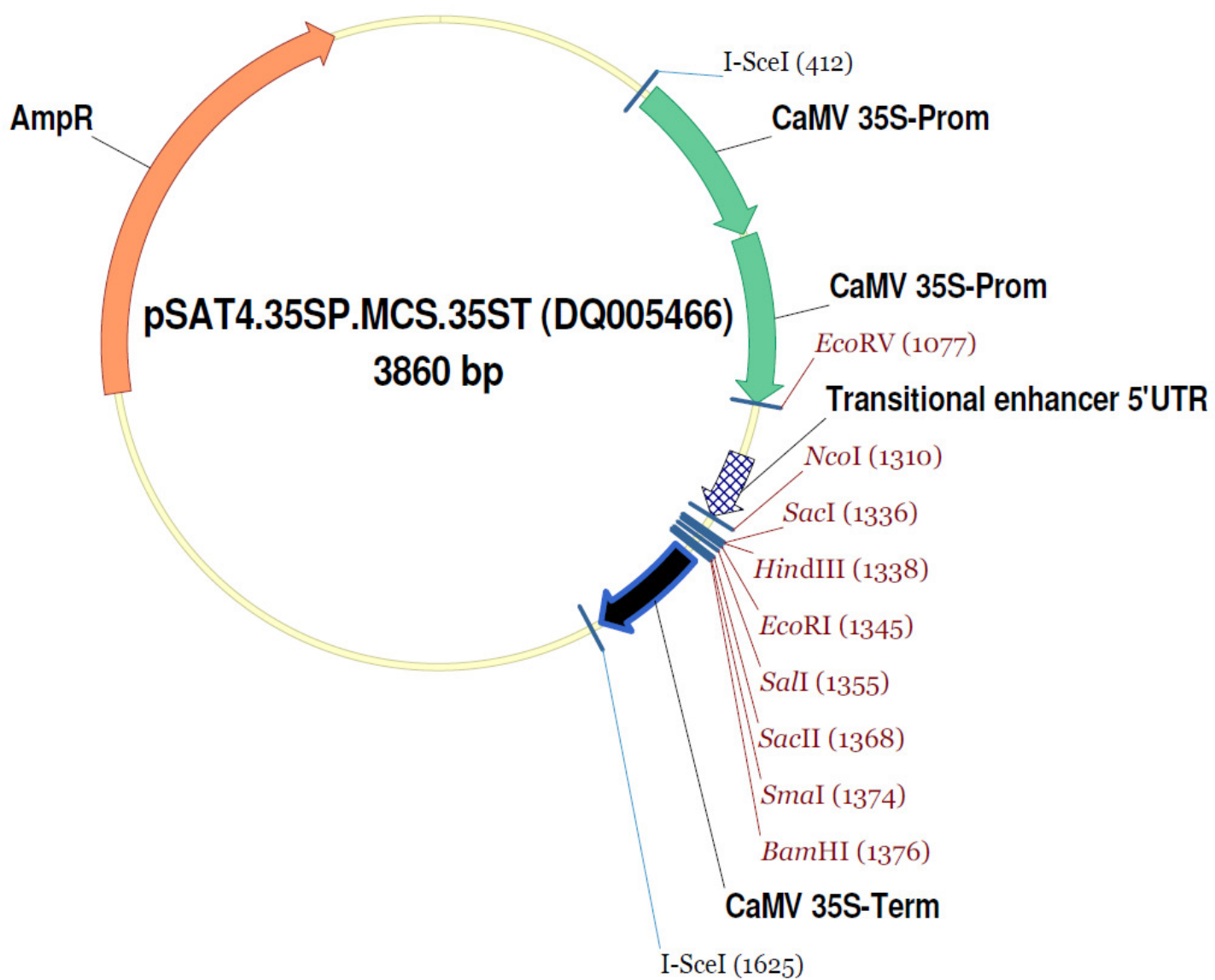


Plasmid map of ectoine genes in pOSM 11 from the saline bacterium *Marinococcus halophilus*.

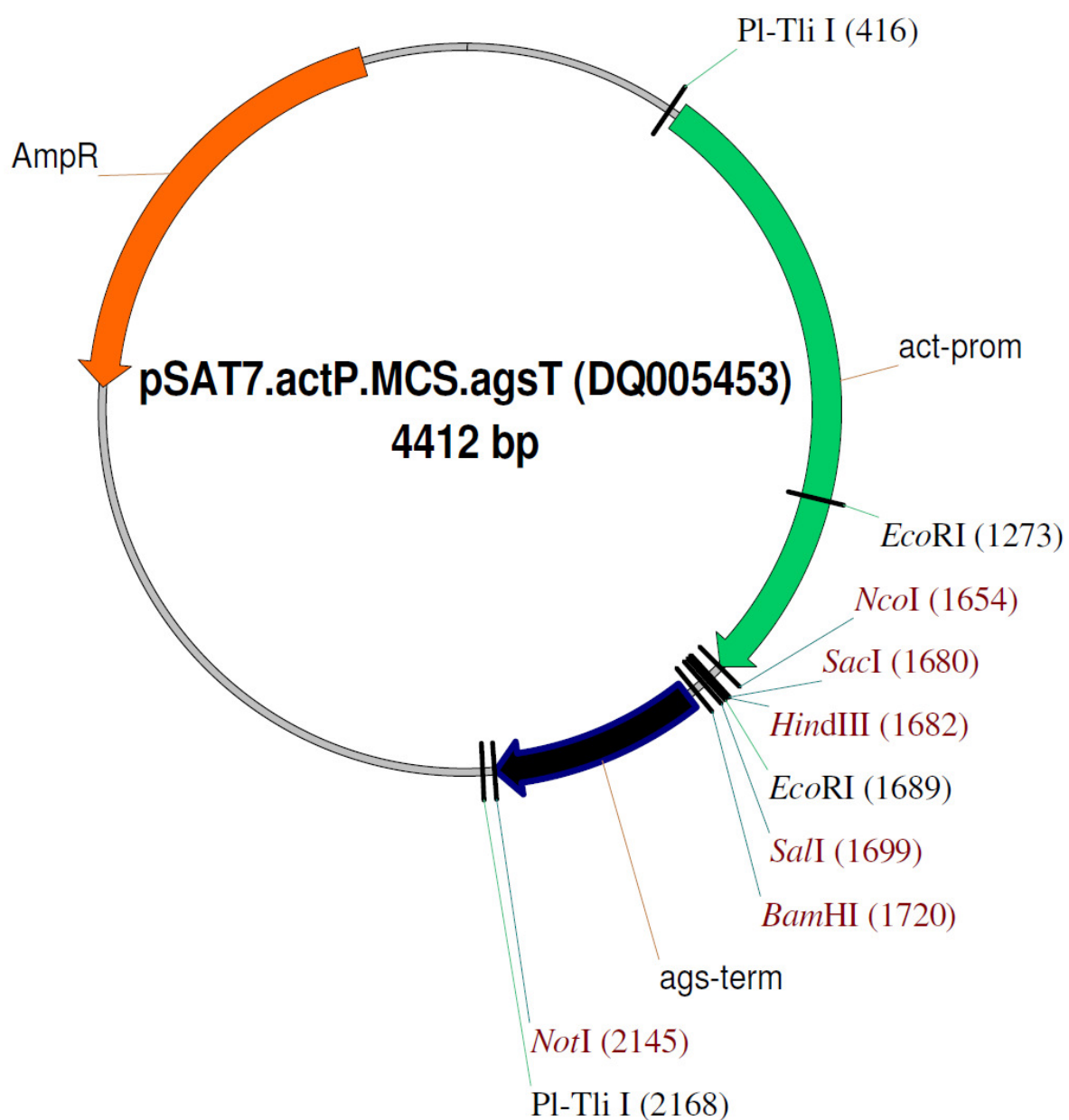
Note here the genes are shown with point mutations created with primers.



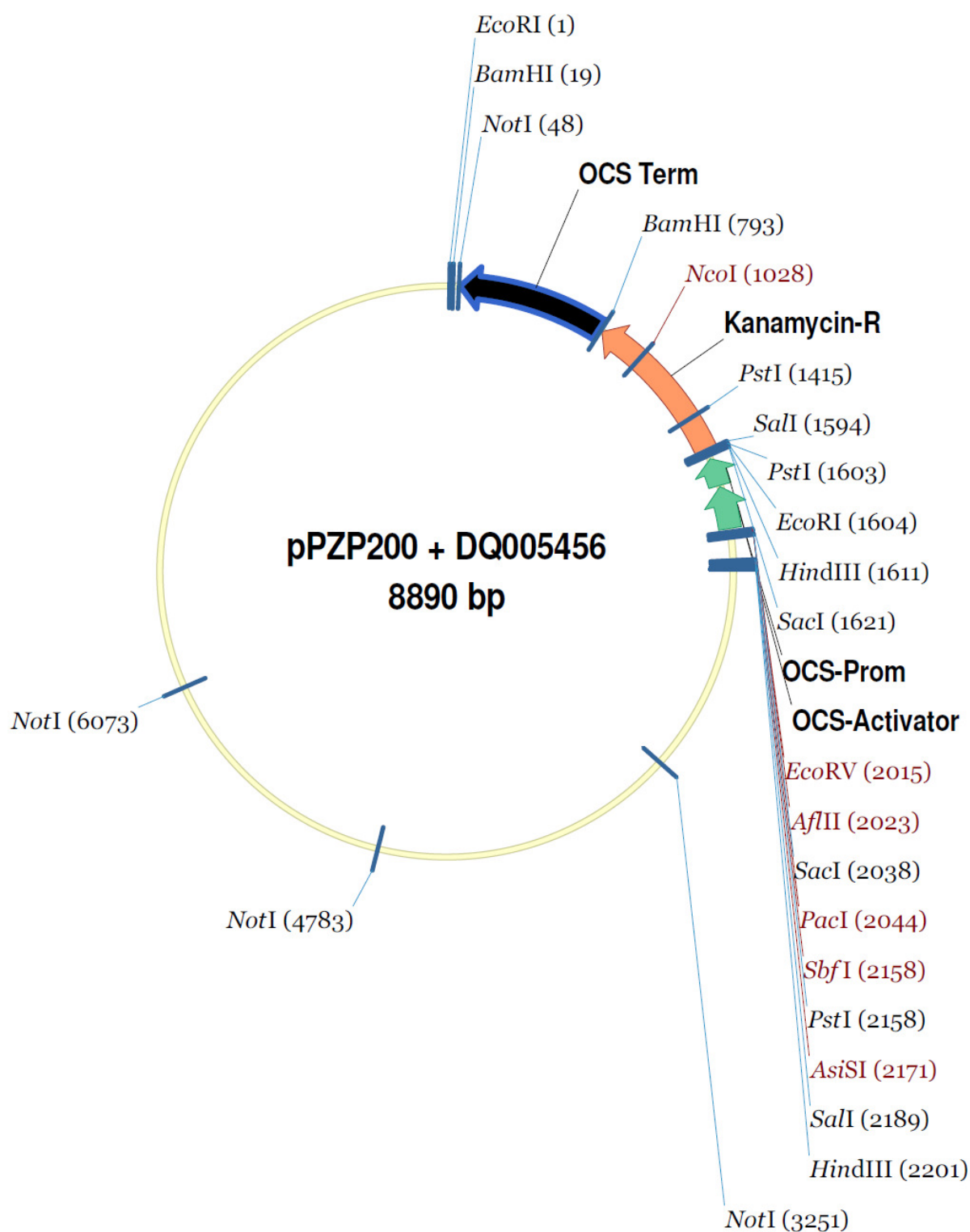
Chung, S.M., Frankman, E.L. and Tzfira, T. A versatile vector system for multiple gene expression in plants. *Trends Plant Sci.* 10 (8), 357-361 (2005) PMID 15993643



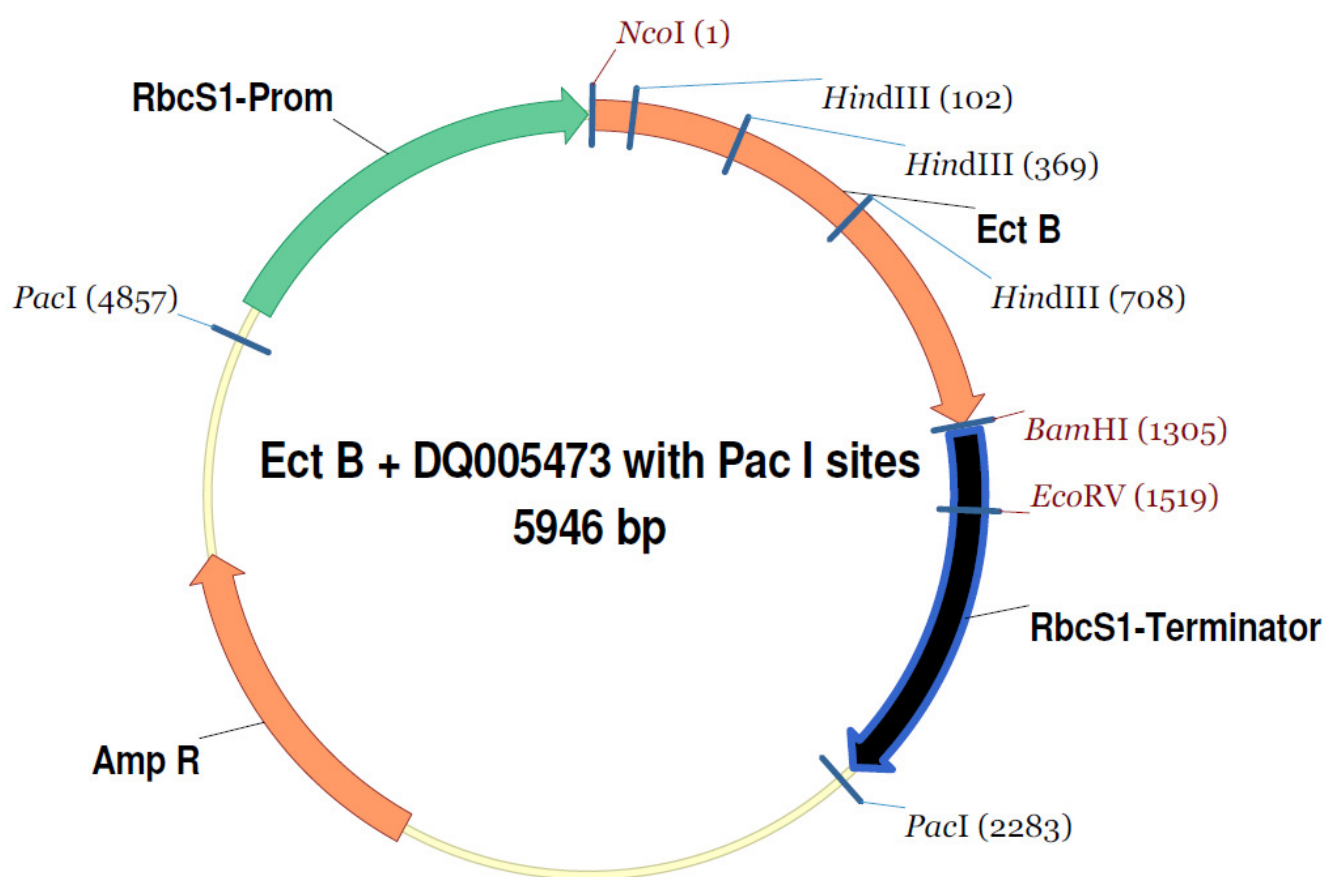
Chung, S.M., Frankman, E.L. and Tzfira, T. A versatile vector system for multiple gene expression in plants. *Trends Plant Sci.* 10 (8), 357-361 (2005) PUBMED 15993643



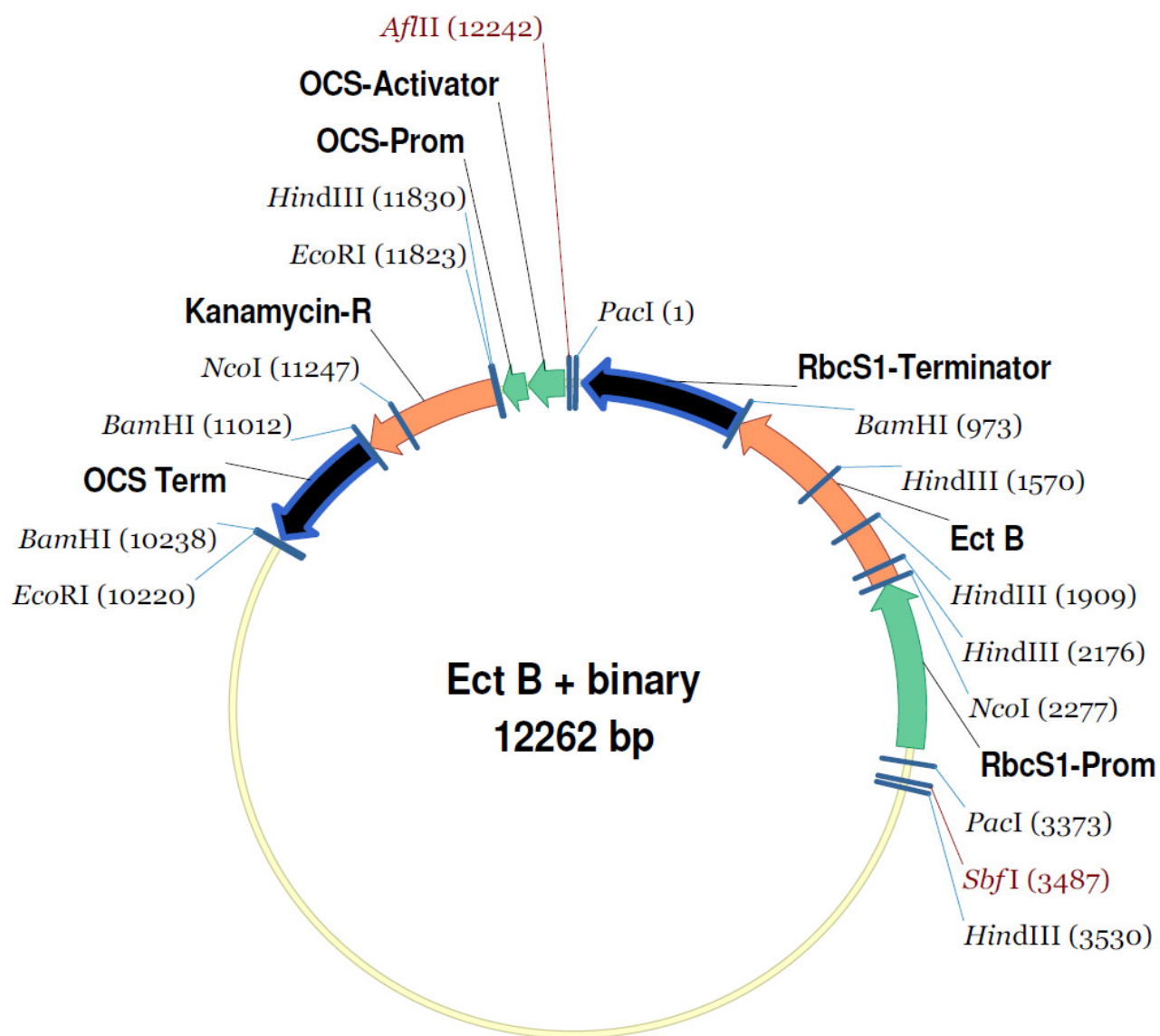
Chung,S.M., Frankman,E.L. and Tzfira,T.A versatile vector system for multiple gene expression in plants.Trends Plant Sci. 10 (8), 357-361 (2005) PUBMED 15993643



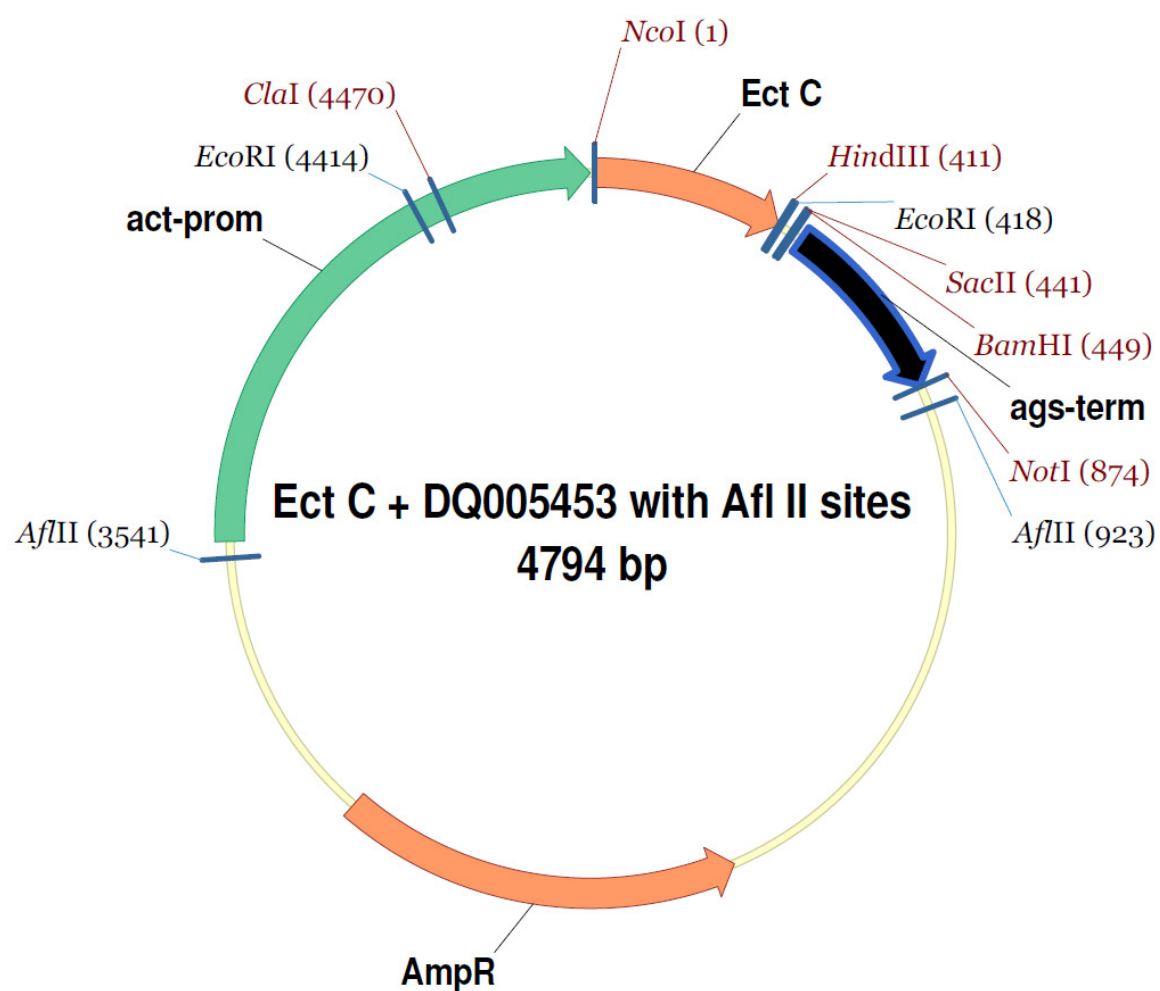
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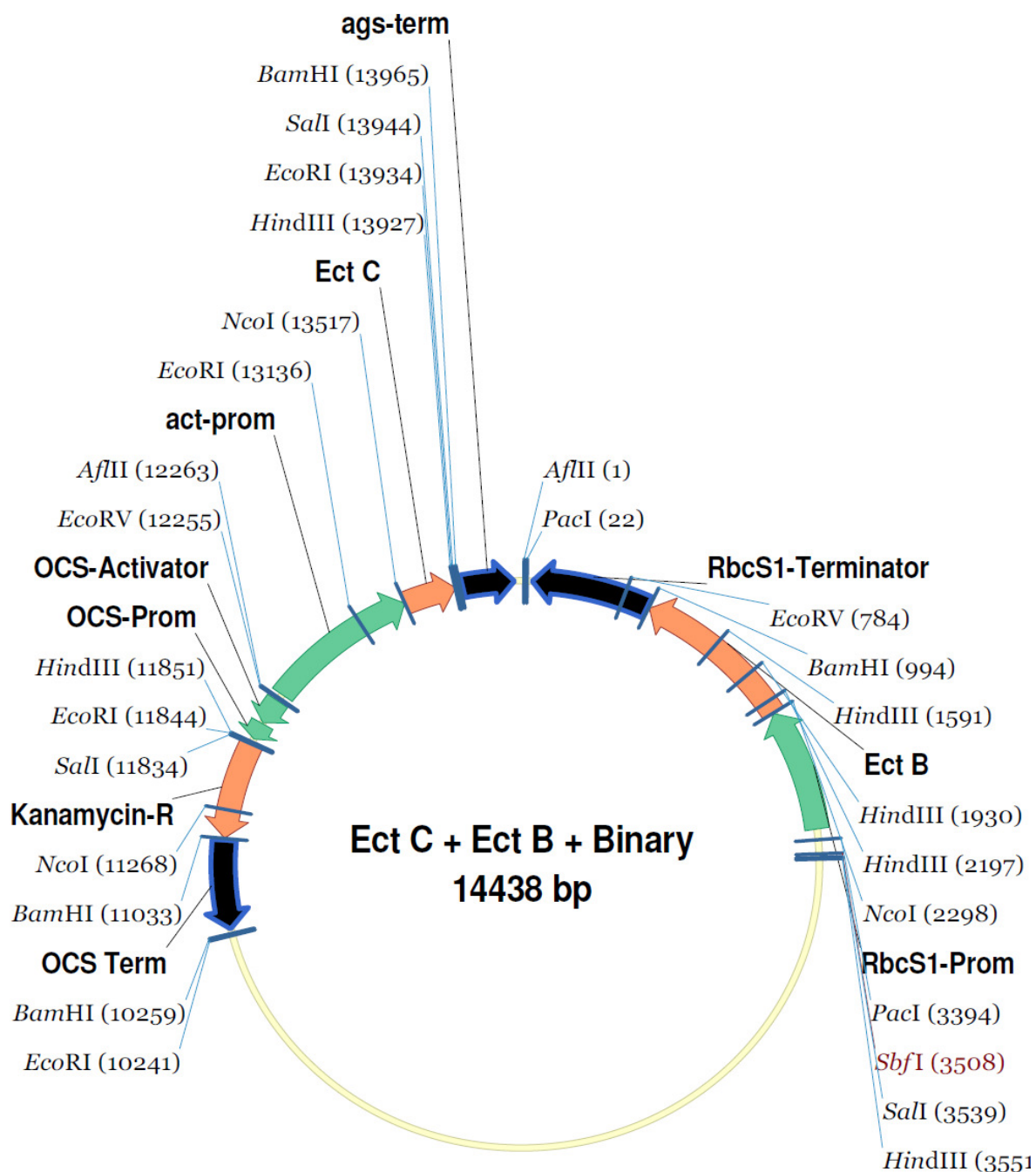
Constructed Ect B + DQ005473 (Ect B cassette) with Pac I restriction sites



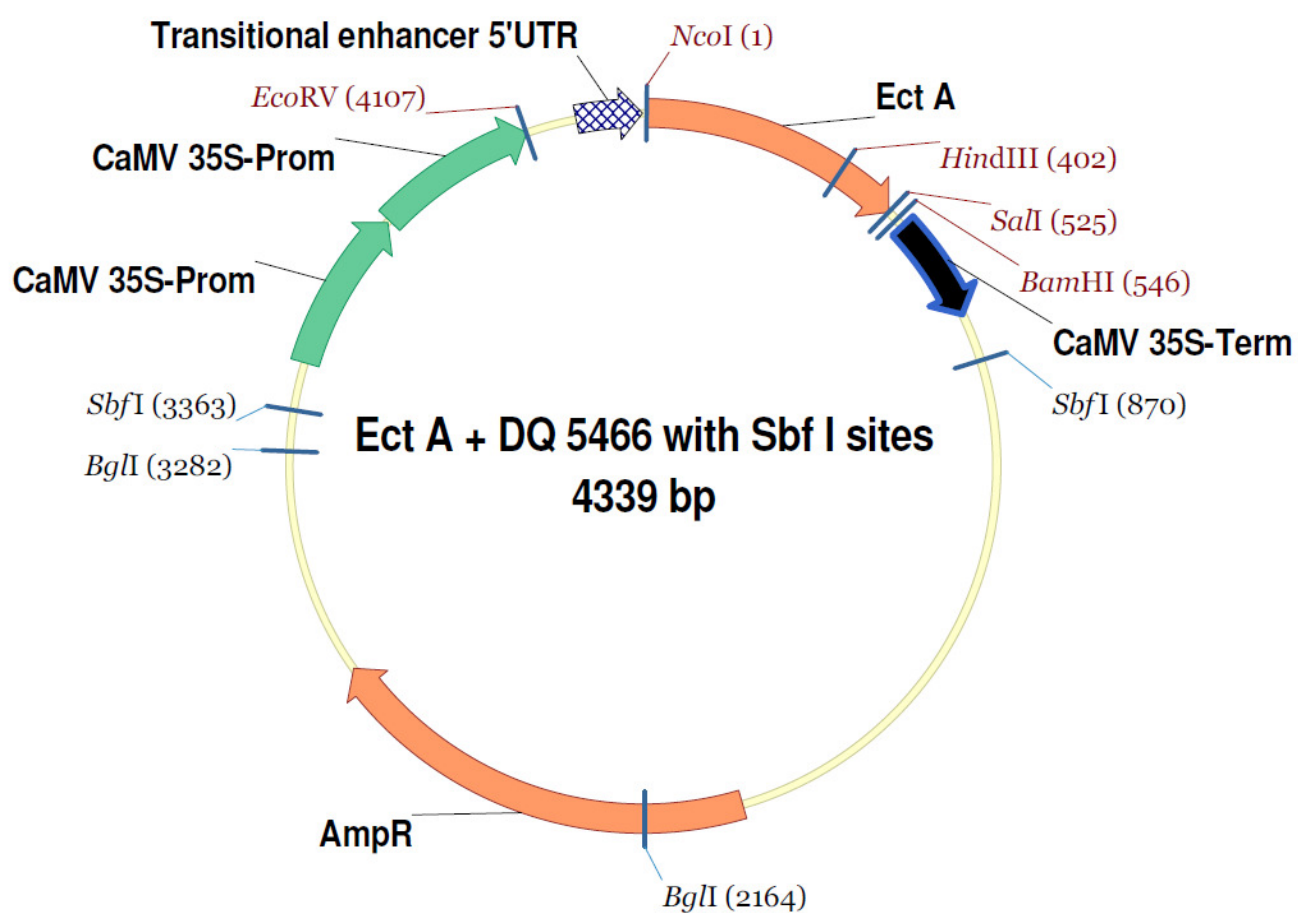
Constructed Ect B cassette in binary vector; pPZP 200 + DQ005456



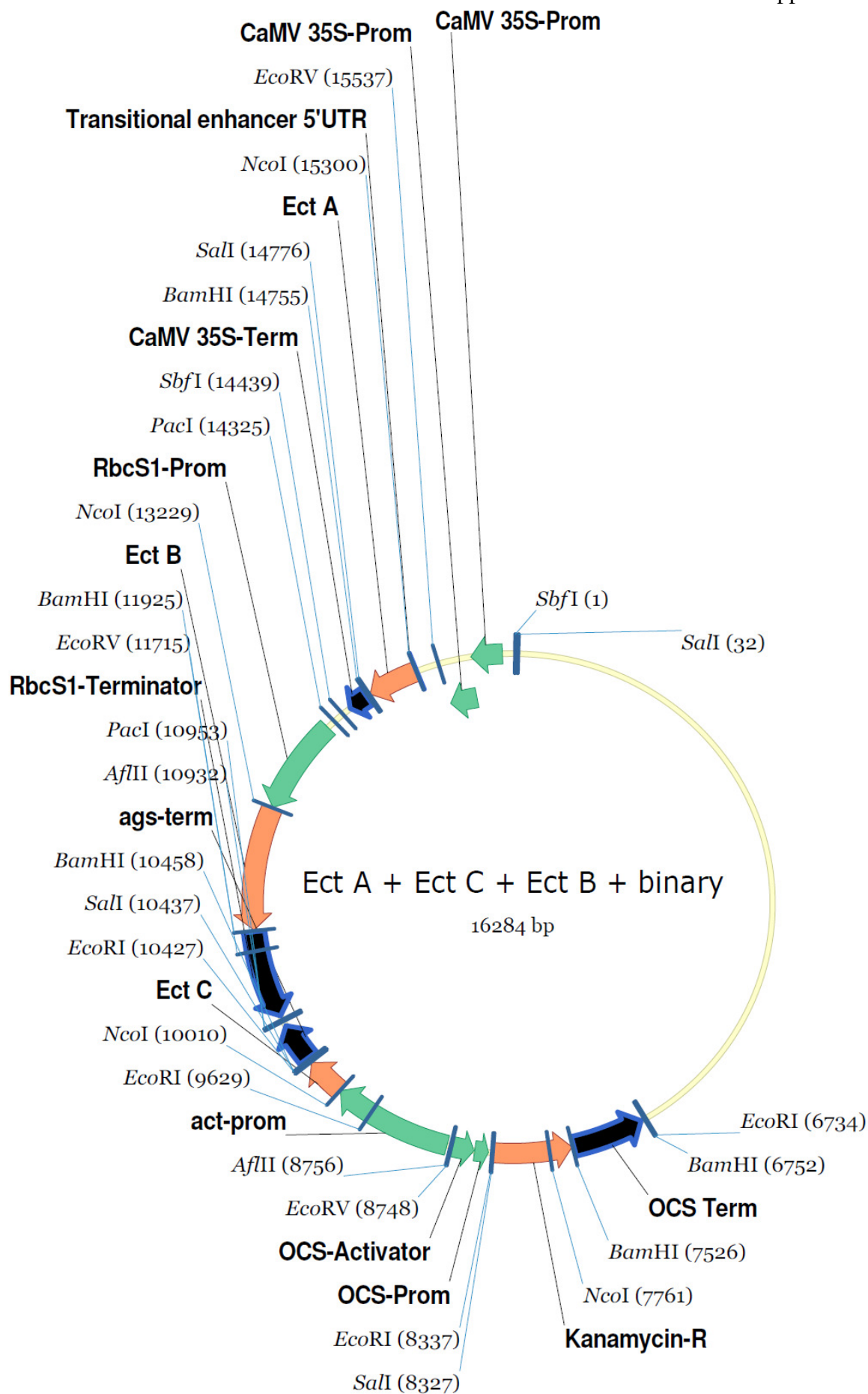
Constructed Ect C+ DQ005453 (Ect C cassette) with Afl II restriction sites



Constructed Ect C cassette + Ect B cassette in binary vector; pPZP 200 + DQ005456



Constructed Ect A + DQ005466 (Ect A cassette) with Sbf I restriction sites



The final constructed binary vector used to transform agrobacteria and plants with Ect A cassette + Ect C cassette + Ect B cassette

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